

Identification of Novel Biomarkers and Pathways for Coronary Artery Calcification in Nondiabetic Patients on Hemodialysis Using Metabolomic Profiling

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Abstract

Background A better understanding of the pathophysiology involving coronary artery calcification (CAC) in patients on hemodialysis (HD) will help to develop new therapies. We sought to identify the differences in metabolomics profiles between patients on HD with and without CAC.

Methods In this case-control study, nested within a cohort of 568 incident patients on HD, the cases were patients without diabetes with a CAC score >100 ($n=51$), and controls were patients without diabetes with a CAC score of zero ($n=48$). We measured 452 serum metabolites in each participant. Metabolites and pathway scores were compared using Mann-Whitney U tests, partial least squares-discriminant analyses, and pathway enrichment analyses.

Results Compared with controls, cases were older (64 ± 13 versus 42 ± 12 years) and were less likely to be Black (51% versus 94%). We identified three metabolites in bile-acid synthesis (chenodeoxycholic, deoxycholic, and glycolithocholic acids) and one pathway (arginine/proline metabolism). After adjusting for demographics, higher levels of chenodeoxycholic, deoxycholic, and glycolithocholic acids were associated with higher odds of having CAC; comparing the third with the first tertile of each bile acid, the OR was 6.34 (95% CI, 1.12 to 36.06), 6.73 (95% CI, 1.20 to 37.82), and 8.53 (95% CI, 1.50 to 48.49), respectively. These associations were no longer significant after further adjustment for coronary artery disease and medication use. Per 1 unit higher in the first principal component score, arginine/proline metabolism was associated with CAC after adjusting for demographics (OR, 1.83; 95% CI, 1.06 to 3.15), and the association remained significant with additional adjustments for statin use (OR, 1.84; 95% CI, 1.04 to 3.27).

Conclusions Among patients on HD without diabetes mellitus, chenodeoxycholic, deoxycholic, and glycolithocholic acids may be potential biomarkers for CAC, and arginine/proline metabolism is a plausible mechanism to study for CAC. These findings need to be confirmed in future studies.

KIDNEY360 2: 279–289, 2021. doi: <https://doi.org/10.34067/KID.0004422020>

Introduction

In patients with ESKD on hemodialysis (HD), coronary artery calcification (CAC) is prevalent and independently predicts the risk of cardiovascular disease, which is the leading cause of death in this population (1–6). The mechanisms of CAC in patients on HD

beyond the passive process of calcium-phosphate precipitation are not well understood. In this hypothesis-generating study, we used metabolomic profiling to identify new biomarkers and pathways for CAC among patients on HD. Metabolomic profiling, which studies small molecules such as amino acids in biologic

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specimens, may provide more insight into the factors involved in cardiovascular calcification. In general, the metabolome is downstream of transcriptional and translational processes and incorporates inputs from diet, environment, and the microbiome—thus, metabolomics reflects a summative assessment of genes, proteins, and exogenous factors (7). In addition, metabolomics has the potential for rapid translational application, because some metabolites may be amenable to therapeutic targeting (8).

Previous studies have examined serum metabolites and cardiovascular mortality in patients on HD (9,10), but none have studied the relationship between serum metabolites and cardiovascular calcification. We performed a case-control study, nested in an existing HD cohort from the Predictors of Arrhythmic and Cardiovascular Risk in ESKD (PACE) study, and compared serum metabolites in patients on HD who were nondiabetic with and without CAC. For metabolites and pathways identified through this analysis, we further explored their relationships with mineral metabolism, calcification inhibitors, and inflammation.

Materials and Methods

Study Design and Population

We conducted a nested, case-control study within PACE, a prospective cohort designed to determine cardiovascular and dialysis-related risk factors in patients on HD (11). PACE was approved by the Johns Hopkins School of Medicine and MedStar Institutional Review Boards. A total of 568 incident patients on HD (*i.e.*, on HD for <6 months) were recruited from 25 freestanding outpatient HD units and two hospital-based outpatient units in Baltimore, Maryland and its surrounding area from 2008 to 2012. CAC was measured at the baseline visit using computed tomography (CT) scanning and was quantified using Agatston score (11). All PACE participants were evaluated for potential inclusion into our metabolomics study. A total of 130 participants who were nondiabetic had serum stored and underwent CAC measurement (Figure 1, Supplemental Methods, Supplemental References; *n* was used to denote the number of participants, and *m* for the number of metabolites). We limited our study to participants without diabetes mellitus to avoid potential confounding effects of diabetes-associated metabolic dysregulation (12). Controls (*n*=48)

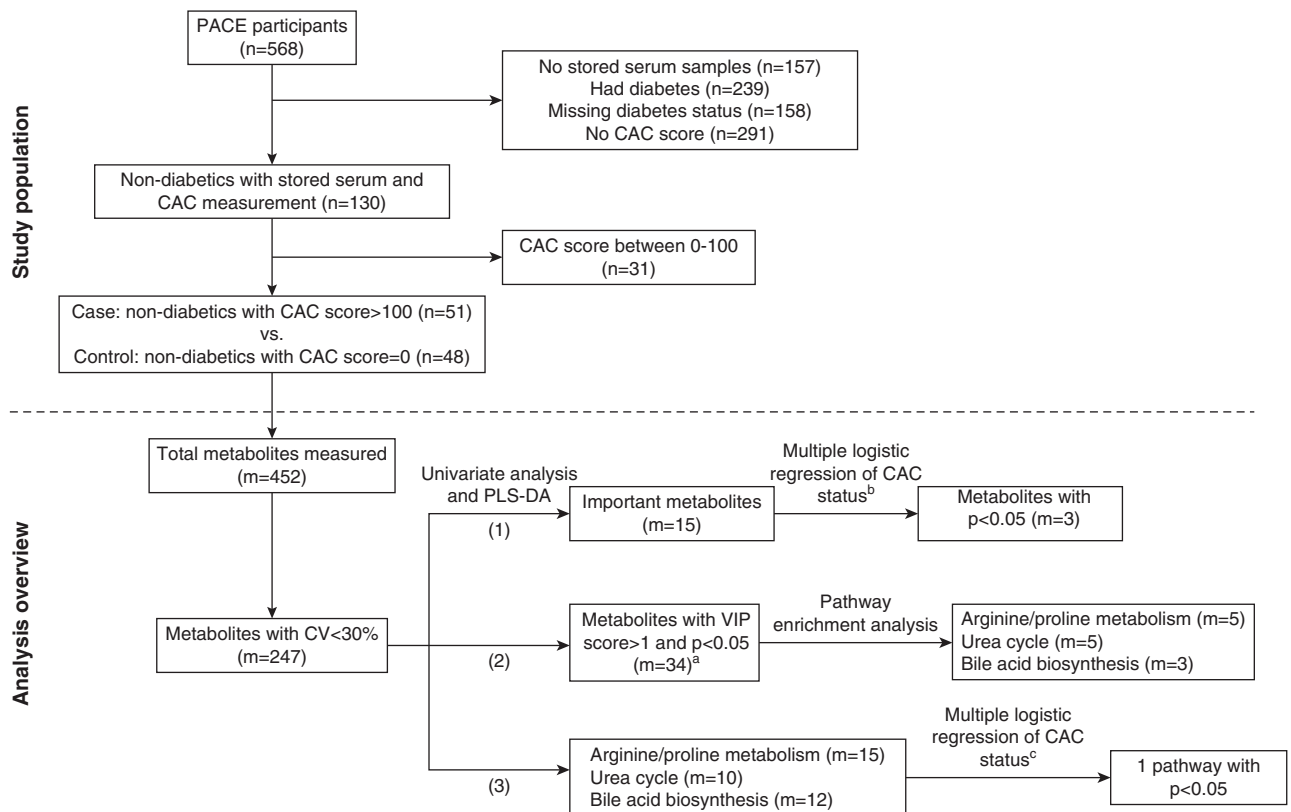


Figure 1. | We conducted a case-control study nested in the Predictors of Arrhythmic and Cardiovascular Risk in ESKD (PACE) cohort and compared serum metabolites and pathway scores between cases and controls. Cases were participants without diabetes with a coronary artery calcification (CAC) score >100 (*n*=51), whereas controls were those with a CAC score of 0 (*n*=48). Metabolites (*m*=247) and pathway scores were compared between cases and controls. We identified three metabolites and one pathway that remained significant after adjusting for age and race. ^aThere were 36 metabolites with variable importance in projection (VIP) score greater than one and *P* value <0.05. Two of these metabolites do not have Human Metabolome Database identification: lumazine (food component) and 3-hydroxybenzaldehyde (chemical), and they were not included in the pathway analysis. ^bAdjusted for age and race using each metabolite. ^cAdjusted for age and race using the first principal component of the pathways. CV, coefficient of variation; *m*, number of metabolites; *n*, number of participants; PLS-DA, partial least squares–discriminant analyses.

were participants with a CAC score of zero. Cases ($n=51$) were patients defined as having a CAC score >100 . The remaining 31 participants with CAC scores between zero and 100 were not included in the study. CT is a sensitive method for detecting coronary calcium, with a sensitivity as high as 99% using a cutoff score of zero; however, its specificity is lower, approximately 65% (13,14). By using a cutoff of 100 to define cases, we increased the specificity to 77% (14). We compared the baseline characteristics of study population with unselected participants in Supplemental Table 1. Cases and controls were not matched.

Measurement of Serum Metabolites

Serum was collected on a non-HD day, at the baseline visit after approximately 8 hours of fasting, and stored at -80°C . Using triple quadrupole mass spectrometry (AB Sciex 6500+ QTRAP; Sciex, Concord, Canada) coupled with a Waters (Milford, MA) Ultra Performance Liquid Chromatography system as previously described (15), we performed widely targeted metabolomics (452 metabolites) in stored serum. A pooled quality-control sample was injected six times and used to calculate the coefficients of variation (CVs). A distribution of CVs is shown in Supplemental Figure 1A. Metabolites with a CV $<30\%$ ($n=247$) were included in the analyses, and their pathways are shown in Supplemental Figure 1B. Missing metabolite values (1%) were replaced by half of the minimum positive value in the original dataset.

Measurement of Covariates

Potential confounders included demographics, education level, smoking history, comorbidities, medication use, dialysis access, and dialysis clearance. Participants' demographic factors (age, sex, and race), education level, smoking history, and medication use were self-reported. Comorbidities, including diabetes mellitus, coronary artery disease (CAD), and hypertension, were adjudicated by a committee of physicians. Dialysis clearance was assessed by single-pool Kt/V. We also measured serum markers of mineral metabolism (calcium, phosphorous, intact parathyroid hormone, C-terminal fibroblast growth factor 23 [FGF-23], and soluble klotho); circulating inhibitors of cardiovascular calcification (osteoprotegerin, dephosphorylated and uncarboxylated matrix glutamate protein [dp-ucMGP], and fetuin-A); secondary calciprotein particle size, and the time of half maximal transformation from primary to secondary calciprotein particle (T_{50}); and high-sensitive C-reactive protein. Details of these measurements are available in Supplemental Methods.

Statistical and Pathway Analyses

Baseline participant characteristics were examined by CAC status using two-sample t test, Mann–Whitney U test, or chi-squared test, as appropriate. Metabolomic data were normalized using auto scaling (centered on the mean and divided by the SD of each metabolite). Figure 1 includes an analysis flowchart to identify significant metabolites and pathways.

First, we compared each metabolite (non-normally distributed) by the CAC status using Mann–Whitney U tests and partial least squares–discriminant analyses (PLS-DA).

For Mann–Whitney U tests, we used a P value threshold of <0.005 and a fold-difference threshold of 1.0 to obtain approximately ten metabolites that differentiated cases from controls. The results were presented in a volcano plot. PLS-DA is commonly used to analyze metabolomics datasets because of its ability to analyze data with high collinearity (16). For PLS-DA, we identified the top 15 metabolites on the basis of the variable importance in project (VIP) scores. For the top metabolites ($m=15$) identified by Mann–Whitney U tests or PLS-DA, we examined the association of each metabolite with CAC using multiple logistic-regression models, adjusting for demographics (age, sex, and race). For the metabolites that had a P value <0.05 after adjusting for demographics ($m=3$; chenodeoxycholic, deoxycholic, and glycolithocholic acids), we generated fully adjusted models using backward elimination. In addition to individual metabolite (in continuous scale) and demographics, backward elimination selected from the following nine covariates: body mass index; smoking history; education level; single-pool Kt/V; CAD; and medication use, including vitamin D therapy, calcium-based phosphate binder, renin-angiotensin-aldosterone system (RAAS) blockade, and statin. The significance threshold to remove a covariate from the models was 0.1. The backward-elimination approach generated the same set of covariates for chenodeoxycholic, deoxycholic, and glycolithocholic acids, and the final full models were adjusted for demographics; CAD; and use of calcium-base phosphate binder, RAAS blockade, and statin. Additionally, we refitted logistic models using tertiles of the three bile acids, and log-transformed the bile acids to examine their correlations with each other using Pearson correlation.

Second, we performed pathway-enrichment analyses using the Small Molecule Pathway Database. To produce robust results, we used the metabolites that met the following three criteria: a VIP score greater than one, P value <0.05 , and available human metabolome database identification ($m=34$). The top three pathways were identified on the basis of the combination of pathway significance and impact. Pathway significance was assessed using global tests with a false discovery rate–adjusted P value threshold of 0.05, and pathway impact was assessed using topology analysis with relative-betweenness centrality.

Third, we tested the associations between the top three pathways and CAC status. To do that, we used principal component analysis and metabolites with CV $<30\%$ ($m=247$) to score the top three pathways identified in step 2. The first principal component (PC1) score of each pathway was then used to represent the pathway. We examined the association between the PC1 of each pathway with CAC using multiple logistic regression. Models were first adjusted for demographics, and then, similar to step 1, we generated fully adjusted models using backward elimination, which selected covariates in addition to individual PC1 of each pathway and demographics. The fully adjusted models were adjusted for demographics and statin use for arginine/proline-metabolism and urea-cycle pathways. For the bile-acid synthesis pathway, models were adjusted for demographics; CAD; and use of calcium-based phosphate binder, RAAS blockade, and statin. For secondary analyses, we used Tobit regression to simultaneously model the presence and severity of CAC, and to examine their

associations with the key metabolites and pathways identified in step 1 and 2 (17). Because CAC score has a right skewed distribution and zero scores, we log-transformed CAC score plus 1, and used left censoring at zero and bootstrap techniques with 999 repetitions.

Lastly, using Spearman rank correlation, we examined the correlations of key metabolites and/or pathways with the markers of mineral metabolism, inflammation, circulating inhibitors of cardiovascular calcification, and properties of calciprotein particle transformation. MetaboAnalyst 4.0 (McGill University, Montreal, Canada) (18) and STATA 14.1 (StataCorp, College Station, TX) were used for statistical and pathway analyses. A two-sided *P* value of <0.05 was considered statistically significant in the multiple logistic and Tobit regression models.

Results

Participant Characteristics

As shown in Table 1, the mean age of participants was 53 ± 16 years, 38% were women, and 72% were Black. All participants had hypertension and 27% had CAD. Among cases, the median CAC score was 466 (interquartile range, 246–981). Compared with controls, cases were older (64 ± 13 versus 42 ± 12 years), less likely to be Black (51% versus 94%), and more likely to have CAD (45% versus 8%). Cases were also less likely to be taking medications that blocked RAAS (36% versus 58%) and were more likely to be on statin therapy (42% versus 13%).

Identification of Significant Metabolites

Using the Mann–Whitney *U* test, and a set threshold of fold difference (1.0) and raw *P* value (0.005), we identified nine metabolites that differed between cases and controls, as shown in the volcano plot (Supplemental Figure 2, Supplemental Table 2). Compared with the controls, bile-acid levels of chenodeoxycholic acid, deoxycholic acid, and glycolithocholic acid were more than two-fold higher in cases (Figure 2). Using PLS-DA, we identified the top 15 metabolites on the basis of VIP scores (Supplemental Figures 3 and 4). The metabolites that were identified by the volcano plot and PLS-DA are summarized in Supplemental Table 2. In addition to the nine metabolites shown in the volcano plot, PLS-DA identified three metabolites involved in arginine/proline metabolism: arginine, ornithine, and succinic acid (Figure 2).

After adjusting for demographics, only bile acids remained significantly associated with CAC status (Supplemental Table 2). Higher levels of chenodeoxycholic, deoxycholic, and glycolithocholic acids were associated with higher odds of CAC. After adjusting for demographics, compared with the participants with bile-acid levels in the first tertile, those in the third tertile had higher odds of CAC with an odds ratio (OR) of 6.34 (95% CI, 1.12 to 36.06; *P*=0.04), 6.73 (95% CI, 1.20 to 37.82; *P*=0.03), and 8.53 (95% CI, 1.50 to 48.49; *P*=0.02) for chenodeoxycholic, deoxycholic, and glycolithocholic acid, respectively (Table 2). However, the associations were no longer significant after additional adjustment for CAD and use of calcium-based phosphate binder, RAAS blockade, and statin.

Chenodeoxycholic, deoxycholic, and glycolithocholic acids were correlated with each other (Supplemental

Figure 5). For secondary analyses, Tobit regression revealed overall similar observations as the logistic regression (Supplemental Table 3). After adjusting for demographics, the difference in the CAC score of those with a CAC score greater than zero (weighted by the probability of having a CAC score greater than zero) and in the probability of having CAC score greater than zero (weighted by the expected value of CAC score if greater than zero) was 0.15 per 1 unit higher in relative intensity of chenodeoxycholic acid (*P*=0.02). The association remained significant after further adjustment for CAD and medication use (*P*=0.03). Deoxycholic and glycolithocholic acids were associated with CAC using Tobit regression after adjusting for demographics, but not in the fully adjusted models.

Identification of Significant Pathways

Pathway-enrichment analyses revealed three top pathways: arginine/proline metabolism, urea cycle, and bile-acid synthesis (Table 3). Metabolites analyzed in these three pathways are shown in Supplemental Table 4. When comparing cases with controls, five metabolites in arginine/proline metabolism, four in urea cycle, and three in bile-acid synthesis had a *P* value <0.05 (Figure 2). L-Arginine, ornithine, and citrulline were present in both arginine/proline-metabolism and urea-cycle pathways.

We used principal component analysis to generate scores for the three pathways. The PC1 score explained 20% of variance in the arginine/proline metabolism, 24% in urea cycle, and 37% in bile-acid synthesis (Supplemental Figure 6). PC1s of arginine/proline metabolism and urea cycle were associated with CAC status, but only PC1 of arginine/proline metabolism remained significant after adjusting for demographics and statin use, with an adjusted OR of 1.84 (95% CI, 1.04 to 3.27) per 1 unit higher of PC1 score (*P*=0.04; Table 4). In secondary analyses using Tobit regression, we found that the presence and severity of CAC were associated with the PC1 of both arginine/proline-metabolism (*P*=0.02) and urea-cycle pathways (*P*=0.04) in the fully adjusted model, but not with PC1 of bile-acid synthesis (Supplemental Table 3).

Correlations with Significant Metabolites and Pathways

We then examined the correlations of bile-acid synthesis and arginine/proline-metabolism pathways and their key metabolites with serum markers of mineral metabolism and inflammation, circulating inhibitors of cardiovascular calcification, and properties of calciprotein particles. Results are shown in a heat map (Figure 3) and in Supplemental Table 5. The PC1 score of bile-acid synthesis was positively correlated with FGF-23 and osteoprotegerin levels, and negatively correlated with T₅₀. Chenodeoxycholic, deoxycholic, and glycolithocholic acids correlated negatively with C-reactive protein, but were not correlated with serum markers of mineral metabolism or circulating inhibitors of calcification. The PC1 score of arginine/proline metabolism correlated positively with levels of serum phosphorous, FGF-23, osteoprotegerin, and dp-ucMGP.

Table 1. Baseline participant characteristics by CAC status

Characteristics	Total (<i>n</i> =99)	Control (CAC Score=0, <i>n</i> =48)	Case (CAC Score >100, <i>n</i> =51)	<i>P</i> Value
Age, yr	53±16	42±12	64±13	<0.001
Women, <i>n</i> (%)	38 (38)	19 (40)	19 (37)	0.81
Black race, <i>n</i> (%)	71 (72)	45 (94)	26 (51)	<0.001
Body mass index, kg/m ²	27±6	28±7	27±5	0.66
History of smoking, <i>n</i> (%)	67 (68)	30 (63)	37 (73)	0.29
Education less than grade 11, <i>n</i> (%)	44 (44)	24 (50)	20 (39)	0.50
Coronary artery disease, <i>n</i> (%)	27 (27)	4 (8)	23 (45)	<0.001
Medication use				
RAAS blockade, <i>n</i> (%)	41 (46)	23 (58)	18 (36)	0.04
Calcium-based phosphate binder, <i>n</i> (%)	37 (37)	15 (31)	22 (43)	0.22
Vitamin D therapy, <i>n</i> (%)	69 (70)	34 (71)	35 (69)	0.81
Statin use, <i>n</i> (%)	27 (27)	6 (13)	21 (42)	0.001
Single-pool Kt/V	1.8±0.3	1.8±0.3	1.9±0.3	0.08
Dialysis access, <i>n</i> (%)				
AVF	32 (32)	12 (25)	20 (39)	0.31
AVG	5 (5)	3 (6)	2 (4)	
Catheter	62 (63)	33 (69)	29 (57)	
Markers of mineral metabolism				
Serum calcium, mg/dl	8.7±0.7	8.7±0.7	8.7±0.7	0.82
Serum phosphorous, mg/dl	5.3±1.2	5.4±1.4	5.2±1.0	0.37
Serum magnesium, mEq/L	1.8±0.2	1.8±0.2	1.7±0.2	0.36
Intact parathyroid hormone, pg/ml	400 (270–581)	402 (293–583)	387 (252–580)	0.49
FGF-23, RU/ml	777 (222–1310)	819 (171–1284)	754 (316–1500)	0.53
Soluble klotho, pg/ml	356 (268–489)	397 (285–524)	306 (263–453)	0.05
Markers of cardiovascular calcification				
Fetuin-A, mg/L	533±193	547±192	519±195	0.48
dp-ucMGP, ppm	1441 (967–2042)	1258 (783–1800)	1734 (1101–2437)	0.02
Osteoprotegerin, pmol/L	10.1±4.8	8.2±3.4	11.8±5.2	<0.001
CPP2 size, nm	289 (192–394)	289 (187–394)	289 (207–394)	0.97
T ₅₀ , min	307 (227–383)	306 (252–378)	315 (213–398)	0.71
C-reactive protein, μg/ml	4.5 (1.7–11.4)	3.4 (1.5–8.7)	5.3 (2.1–17.1)	0.18

If normally distributed, values for continuous variables with normal distribution are provided as mean±SD and were tested with two-sample *t* test. Otherwise, they are provided as median (interquartile range) and were tested using Mann–Whitney test. Categorical variables are presented as absolute number with percentage and were tested with chi-square test. CAC, coronary artery calcification; RAAS, renin-angiotensin-aldosterone system; AVF, arteriovenous fistula; AVG, arteriovenous graft; FGF-23, fibroblast growth factor23; dp-ucMGP, dephosphorylated and uncarboxylated matrix glutamate protein; CPP2, secondary calciprotein particle; T₅₀, time of half maximal transformation from primary to secondary calciprotein particle.

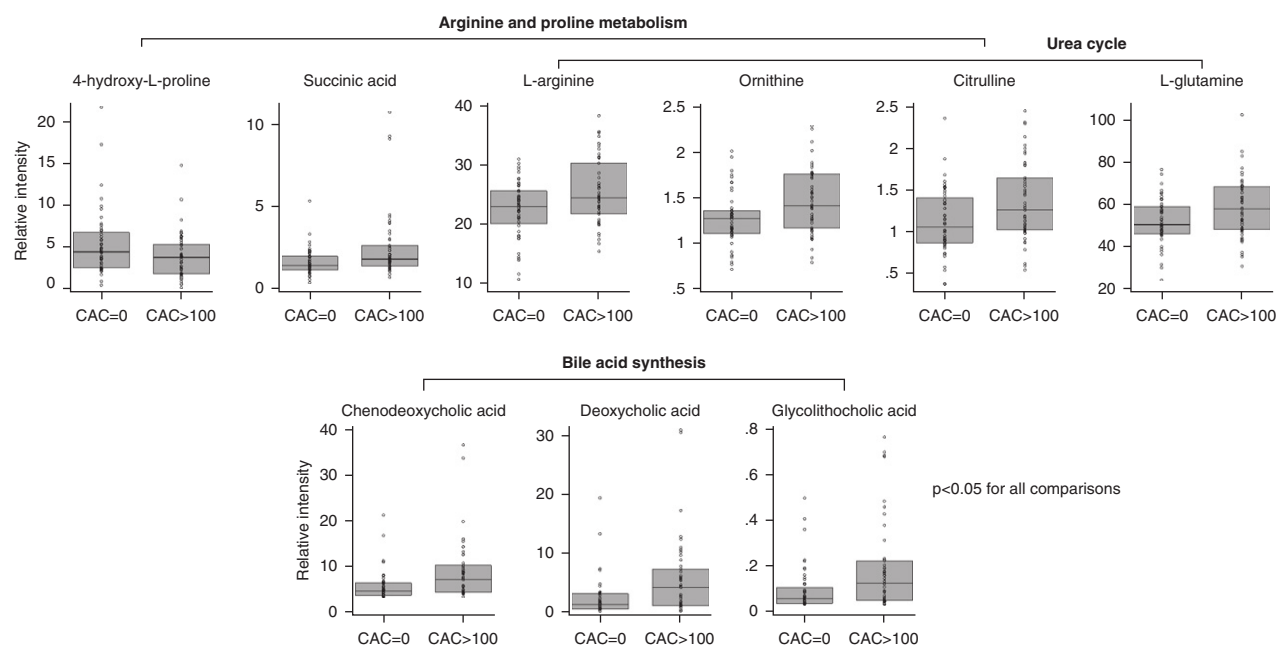


Figure 2. Cases and controls had different levels of selected metabolites in arginine and proline metabolism, urea cycle, and bile-acid synthesis. Mann–Whitney *U* tests were used to compare metabolites between cases (CAC > 100) and controls (CAC = 0). Metabolites with a *P* value < 0.05 are presented above. L-Arginine (fold difference, 1.14; *P* = 0.03), ornithine (fold difference, 1.17; *P* = 0.008), and citrulline (fold difference, 1.21; *P* = 0.01) are in both arginine and proline metabolism and urea cycle. 4-Hydroxy-L-proline (fold difference, 0.74; *P* = 0.04) and succinic acid (fold difference, 1.44; *P* = 0.005) are only in arginine and proline metabolism, whereas L-glutamine (fold difference, 1.12; *P* = 0.02) is only in the urea-cycle pathway. Chenodeoxycholic acid (fold difference, 2.33; *P* = 0.002), deoxycholic acid (fold difference, 2.32; *P* = 0.001), and glycolithocholic acid (fold difference, 2.44; *P* = 0.004) are in the bile-acid synthesis pathway. Box plots represent median and interquartile range.

Discussion

In patients with ESKD on HD, CAC is a prevalent and independent predictor of cardiovascular morbidity and mortality (2–6,19). In this nested, case-control study, we

compared metabolites and pathway scores in 99 incident patients on HD who were nondiabetic with a CAC score > 100 and a CAC score of zero. Compared with those with a CAC score of zero, patients with a CAC score > 100 have

Table 2. Logistic regression of CAC status with chenodeoxycholic, deoxycholic, and glycolithocholic acids

Metabolites	Cases (n)	Unadjusted		Adjusted for Demographics		Fully Adjusted	
		OR (95% CI)	P	OR (95% CI)	P	OR (95% CI)	P
Chenodeoxycholic acid							
Continuous ^a	—	1.17 (1.04 to 1.33)	0.01	1.18 (1.01 to 1.38)	0.04	1.14 (0.97 to 1.34)	0.11
Tertile 1	12	Reference		Reference		Reference	
Tertile 2	13	1.14 (0.42 to 3.08)	0.80	0.84 (0.17 to 4.19)	0.83	0.04 (0.001 to 1.03)	0.05
Tertile 3	26	6.5 (2.17 to 19.43)	0.001	6.34 (1.12 to 36.06)	0.04	1.23 (0.11 to 13.26)	0.87
Deoxycholic acid							
Continuous ^a	—	1.18 (1.04 to 1.34)	0.01	1.17 (1.00 to 1.37)	0.049	1.14 (0.97 to 1.33)	0.10
Tertile 1	11	Reference		Reference		Reference	
Tertile 2	14	1.47 (0.54 to 4.01)	0.45	0.93 (0.19 to 4.61)	0.93	0.05 (0.002 to 1.28)	0.07
Tertile 3	26	7.43 (2.46 to 22.42)	<0.001	6.73 (1.20 to 37.82)	0.03	1.58 (0.16 to 15.66)	0.69
Glycolithocholic acid							
Continuous ^a	—	1.05 (1.01 to 1.09)	0.01	1.07 (1.00 to 1.13)	0.04	1.04 (0.97 to 1.12)	0.27
Tertile 1	11	Reference		Reference		Reference	
Tertile 2	16	1.88 (0.70 to 5.09)	0.21	1.60 (0.31 to 8.10)	0.57	1.45 (0.16 to 13.52)	0.74
Tertile 3	24	5.33 (1.86 to 15.30)	0.002	8.53 (1.50 to 48.49)	0.02	3.78 (0.32 to 44.84)	0.29

n = 33 in each tertile. Demographics included age, sex, and race. Full models were adjusted for demographics; coronary artery disease; and use of calcium-based phosphate binder, renin-angiotensin-aldosterone system blockade, and statin for all three bile acids. CAC, coronary artery calcification; OR, odds ratio.

^aUnits for continuous variables were 1 unit in relative intensity for chenodeoxycholic acid and deoxycholic acid, and 0.01 unit in relative intensity for glycolithocholic acid.

Table 3. Impact and significance of arginine/proline metabolism, urea cycle, and bile-acid synthesis

Pathway	Metabolites in SMPD Pathway (m)	Metabolites Analyzed (m)	Pathway Impact	FDR-Adjusted <i>P</i>
Arginine and proline metabolism	53	15	0.21	5.8×10^{-4}
Urea cycle	29	10	0.23	0.002
Bile-acid synthesis	65	12	0.01	0.005

For pathway-enrichment analyses, we used SMPD and included 34 metabolites that had a variable importance in projection score greater than one and *P* value <0.05. Global tests were used to assess the significance of pathways, and topology analysis with relative betweenness centrality was used to assess pathway impact. SMPD, Small Molecule Pathway Database; FDR, false discovery rate.

more than three times the risk of coronary events (13,20). We identified three metabolites in bile-acid synthesis (chenodeoxycholic, deoxycholic, and glycolithocholic acids) and one metabolic pathway (arginine/proline metabolism) that were associated with CAC.

We found that higher levels of chenodeoxycholic, deoxycholic, and glycolithocholic acids were associated with CAC after adjusting for demographics. Similarly, in a study of patients with predialysis CKD, higher serum deoxycholic acid levels were associated with greater CAC after adjusting for demographics, comorbidities, and kidney function (21). However, after we further adjusted for comorbidities and medication use, the association between bile acids and CAC status was no longer significant. The lack of association in the fully adjusted logistic models could be due to small sample size. In the sensitivity analyses using Tobit regression, chenodeoxycholic acid remained significant after adjusting for demographics, CAD, and medication use. Compared with logistic regression, Tobit regression is more likely to identify predictors for cardiovascular calcification (17).

Elevated bile-acid levels may reflect disruption of intestinal barrier function or alteration in the intestinal microbiome, making them potential candidates as biomarkers for CAC in patients on HD. Bile acids are important for intestinal nutrient absorption and biliary secretion of lipid and toxic metabolites (Supplemental Figure 7A) (22). Chenodeoxycholic acid is a primary bile acid, because it is directly synthesized from cholesterol in liver (23). After synthesis, chenodeoxycholic acid is secreted into the intestinal tract, where it is converted by intestinal bacteria to lithocholic acid, a secondary bile acid (24). Conjugation of lithocholic acid with glycine forms glycolithocholic acid, increasing solubility and decreasing toxicity (22,25). Cholic acid, another primary bile acid, is also converted in the intestinal tract to deoxycholic acid, a secondary bile acid. After bile acids are secreted into the intestinal tract, they are reabsorbed in the ileum, or by portal circulation, back to the liver. This enterohepatic circulation of bile acids is generally highly efficient (22). Patients on HD may have a disrupted intestinal barrier, which results in translocation of bile acids, endotoxins, and bacterial metabolites in the systemic circulation (26–28). These translocated metabolites and toxins may then contribute to chronic inflammation and, ultimately, to cardiovascular disease (29–32).

Animal studies have shown that bile acids may have direct effects on cardiovascular calcification *via* the farnesoid

X receptor, a bile acid nuclear receptor (33,34). In our study, bile-acid synthesis was one of the top three pathways identified by the pathway-enrichment analyses, but its pathway impact was low compared with that of arginine/proline metabolism and urea cycle (0.01 versus 0.21 and 0.23), and the association between the bile-acid synthesis pathway and CAC status was not significant. We also did not observe any significant correlation of bile acids with serum markers of mineral metabolism or circulating inhibitors of calcification. Our findings do not support a causal relationship between bile-acid synthesis and the development of CAC.

We found that arginine/proline metabolism was associated with CAC in both fully adjusted logistic and Tobit regression models. The urea-cycle pathway has three common metabolites with arginine/proline metabolism (*i.e.*, L-arginine, ornithine, and citrulline), and was associated with CAC only in Tobit regression. Supplemental Figure 7B illustrates the intracellular arginine/proline metabolism, one of the central pathways for the biosynthesis of arginine and proline from glutamine (35–37). Compared with controls, cases had higher levels of L-arginine, ornithine, citrulline, and succinic acid, and lower levels of 4-hydroxy-L-proline. None of the amino acids were associated with CAC after adjusting for demographics, but their relative betweenness centrality resulted in a high pathway impact. Assuming intracellular metabolism of these amino acids mirrors their extracellular metabolism and serum levels, their directionality seems to suggest there was an increased synthesis of L-arginine and decreased metabolism of proline in participants with CAC.

Patients on HD with CAC might have increased synthesis of L-arginine and decreased metabolism of proline to counteract the calcifying milieu. There are few studies that examined the effect of arginine on cardiovascular calcification. In an adenine-induced model of renal failure and arterial calcification, dietary L-arginine supplementation suppressed arterial calcification in seven out of ten rats (38). The exact mechanism of how arginine may attenuate arterial calcification is unclear. *In vitro*, L-arginine attenuated precipitation of calcium and phosphate (38). Arginine is a precursor for nitric oxide (Supplemental Figure 7B), and its effect on arterial calcification may be mediated by nitric oxide (39). To the best of our knowledge, no animal study has examined the effect of L-proline on arterial calcification. In cell culture, L-proline inhibited the apoptosis of human vascular smooth muscle cells induced by calcium and phosphate (38). Patients with CKD can develop both intimal

Table 4. Logistic regression of CAC status with arginine/proline metabolism, urea cycle, and bile-acid synthesis

Pathway	Unadjusted ^a		Adjusted for Demographics		Fully Adjusted	
	OR (95% CI)	P	OR (95% CI)	P	OR (95% CI)	P
Arginine/proline metabolism	1.43 (1.06 to 1.92)	0.02	1.83 (1.06 to 3.15)	0.03	1.84 (1.04 to 3.27)	0.04
Urea cycle	1.93 (1.34 to 2.80)	<0.001	1.58 (0.93 to 2.69)	0.09	1.66 (0.94 to 2.91)	0.08
Bile-acid synthesis	0.99 (0.82 to 1.20)	0.92	1.09 (0.84 to 1.41)	0.51	1.64 (0.97 to 2.75)	0.06

Demographics included age, sex, and race. Full models were adjusted for demographics and statin use for arginine/proline metabolism and urea cycle pathways; for the bile-acid synthesis pathway, full models were adjusted for demographics; coronary artery disease; and use of calcium-based phosphate binder, RAAS blockade, and statin. CAC, coronary artery calcification; OR, odds ratio; RAAS, renin-angiotensin-aldosterone system.

^aPer 1 unit higher in the first principal component score of each pathway.

calcification, which is an indicator of atherosclerosis, and medial calcification, which is characterized by diffuse calcium and phosphate deposition. Unfortunately, CT scans cannot differentiate between intimal and medial calcification. Altered arginine/proline metabolism may reflect atherosclerosis rather than mineralization of calcium and phosphate. In rabbits fed with a high-cholesterol diet, arginine supplementation limited the development of atherosclerosis (40). In our exploratory analyses, we found that arginine/proline metabolism was positively correlated with serum phosphorous, FGF-23, osteoprotegerin, and dp-ucMGP. Arginine/proline metabolism may serve as a scaffold on which a variety of regulatory mechanisms for arterial calcification are integrated. Although controversial, arginine supplementation has been used in a wide variety of conditions, such as

hypertension and erectile dysfunction (41). Because the arginine/proline metabolic pathway could be a potential therapeutic target, the role of arginine in arterial calcification merits further investigation.

Our study has several limitations. Because metabolites and CAC were both measured at baseline, the temporal relationship between serum metabolites and CAC could not be studied, thus limiting the inference of potential causal relationships. We were not able to address all potential or unmeasured confounders. Diabetes and hypertension are major potential confounders because both are associated with arterial calcification and metabolic dysregulation (12,42,43). All participants in our study were incident to HD, nondiabetic, and diagnosed with hypertension. Liver and residual renal function may influence the association of

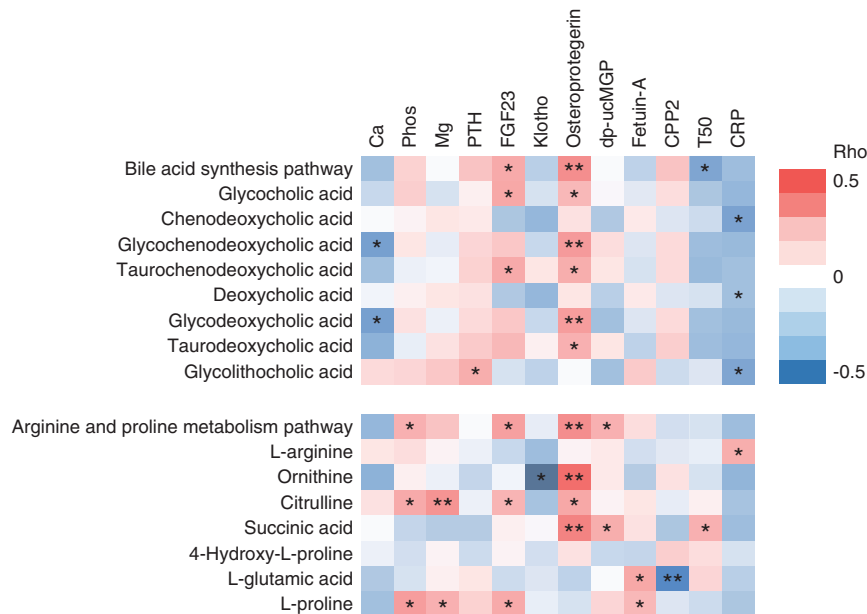


Figure 3. | Correlations of bile-acid synthesis and arginine/proline-metabolism pathways and their key metabolites with serum markers of mineral metabolism (Ca, Phos, Mg, PTH, FGF23, klotho), circulating inhibitors of calcification (osteoprotegerin, dp-ucMGP, fetuin-A, CPP2, T₅₀), and inflammation (CRP). The first principal component scores were used to represent the pathways. Heat map shows Spearman rank correlations. Correlation strength is represented by color bar (red, positive correlation; white, no correlation; blue, negative correlation). **P*<0.05; ***P*<0.005. Ca, calcium; CPP2, secondary calciprotein particle; CRP, C-reactive protein; dp-ucMGP, dephosphorylated and uncarboxylated matrix glutamate protein; FGF-23, fibroblast growth factor-23; klotho, soluble klotho; Mg, magnesium; phos, phosphorous; PTH, intact parathyroid hormone; T₅₀, time of half maximal transformation from primary to secondary calciprotein particle.

serum metabolites with CAC. Unfortunately, liver-function tests were not available in PACE, and only approximately a third of the study population had available data on residual renal function. Due to the limitation in sample size, we did not include residual renal function in our analyses. Last, the study cohort is not representative of the ESKD population in the United States and may need validation in a larger and more diverse ESKD population.

Our study has several strengths. First, we studied the key metabolites and pathways in detail in regard to their relationships with serum markers of mineral metabolism, inflammation, and circulating inhibitors of calcification. Our findings provide an important framework for future studies that investigate the roles of these novel metabolites and arginine/proline metabolism pathway on CAC. Second, all samples were collected after 8 hours of fasting, thus minimizing the effects of diet on metabolites (44). Third, by focusing on incident patients on HD who had similar characteristics, including the status of diabetes and hypertension, we minimized potential residual confounding.

In this nested, case-control study of incident patients on HD who were nondiabetic, we used a relatively unbiased approach of metabolomics profiling and identified three novel metabolites (chenodeoxycholic, deoxycholic, and glycolithocholic acids) and one pathway (arginine/proline metabolism) that were associated with CAC. Chenodeoxycholic, deoxycholic, and glycolithocholic acids may be potential serum biomarkers for CAC, and arginine and proline metabolism may emerge as a new pathway in the pathogenesis of CAC and could be a potential treatment target. Our findings provide new insight into pathophysiology of CAC in patients on HD and lay an important framework for future studies that investigate the roles of bile acids and arginine/proline metabolism on CAC.

Disclosures

D.A. Bushinsky is a consultant for Amgen, Relypsa/Vifor/Fresenius, Sanofi Genzyme, and Tricida, and has an equity interest in Amgen and Tricida. All remaining authors have nothing to disclose.

Funding

The research described was supported by the National Center for Advancing Translational Science grant UL1 TR002556. The PACE study was funded by National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK) grants R01 DK072367 and DK090070 and the National Kidney Foundation Serving Maryland and Delaware. W. Chen is supported by the American Society of Nephrology Carl W. Gottschalk Research Grant and NIDDK grant K23 DK114476. D. Bushinsky is supported by NIDDK grant R01 DK075462. R. S. Parekh is supported by the Canada Research Chair in Chronic Kidney Disease Epidemiology. D. Riascos-Bernal is supported by the American Heart Association career development award 19CDA34660217. N. Sibinga is supported by National Heart, Lung, and Blood Institute grants R01 HL133861 and R01 HL149921, American Heart Association grant 19TPA34890070, and the Irma T. Hirschl-Monique Weill-Caulier Charitable Trust. The Stable Isotope and Metabolomics Core Facility of the Diabetes Research and Training Center of the Albert Einstein College of Medicine is supported by National Cancer Institute grant P60DK020541.

Acknowledgments

We thank participants, nephrologists, and staff of the DaVita and MedStar dialysis units in the Baltimore region who contributed to the PACE study.

Each author contributed important intellectual content during manuscript drafting or revision and accepts accountability for the overall work by ensuring that questions pertaining to the accuracy or integrity of any portion of the work are appropriately investigated and resolved.

Author Contributions

D.A. Bushinsky, W. Chen, Y. Chen, R.F. Dubin, J. Fitzpatrick, B.G. Jaar, R.S. Parekh, Y. Qiu, D.F. Riascos-Bernal, N.E.S. Sibinga, S.M. Sozio, and T.T. Wu reviewed and edited the manuscript; D.A. Bushinsky, W. Chen, R.F. Dubin, J. Fitzpatrick, I.J. Kurland, R.S. Parekh, Y. Qiu, and N.E.S. Sibinga were responsible for methodology; D.A. Bushinsky, Y. Chen, R.S. Parekh, D.F. Riascos-Bernal, and N.E.S. Sibinga provided supervision; D.A. Bushinsky, W. Chen, R.S. Parekh, D.F. Riascos-Bernal, N.E.S. Sibinga, and S.M. Sozio conceptualized the study; W. Chen was responsible for software and visualization; W. Chen, Y. Chen, and R.S. Parekh were responsible for resources; W. Chen, I.J. Kurland, R.S. Parekh, and Y. Qiu were responsible for investigation; W. Chen, M.M. Estrella, J. Fitzpatrick, B.G. Jaar, R.S. Parekh, and S.M. Sozio were responsible for data curation; W. Chen, I.J. Kurland, and Y. Qiu were responsible for project administration; W. Chen and N.E.S. Sibinga wrote the original draft; W. Chen was responsible for funding acquisition; and W. Chen and T.T. Wu were responsible for formal analysis and validation.

Supplemental Material

This article contains the following supplemental material online at <http://kidney360.asnjournals.org/lookup/suppl/doi:10.34067/KID.K3602020000442/DCSupplemental>.

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Supplemental Figure 7. Bile acid synthesis and intracellular arginine and proline metabolism, simplified version.

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Received: July 17, 2020 Accepted: December 2, 2020

Supplemental Material

Title: Identification of Novel Biomarkers and Pathways for Coronary Artery Calcification in Non-diabetic Patients on Hemodialysis Using Metabolomic Profiling

AUTHORS

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References

SUPPLEMENTAL METHODS

Selection of study population

In the PACE cohort (n=568), diabetes status and blood samples were not available in 158 participants because these participants did not complete the study visit. Every participant in the PACE cohort had hypertension. In current study, we compared the serum metabolites of non-diabetics with a CAC score >100 (case) with non-diabetics with a CAC score of 0 (control). Compared to the study population, participants with diabetes were older (56 ± 11 vs. 53 ± 16 years) and more likely to have CAD (45% vs. 27%; Table S1). Participants without available CAC scores (76% had diabetes) were more likely to have CAD (40%) compared to the study population. Non-diabetic participants with a CAC score between 0-100 had similar demographic characteristics with the study population.

Details on CAC measurement

CAC was measured at the baseline visit by computed tomography (Toshiba Aquilon One, Japan), acquired prospectively in synchrony with the electrocardiogram at 70-80% RR interval, near the end of diastole and before atrial contraction.¹ Coronary calcium was quantified by Agatston score. CAC scores were assessed and then validated by a second reviewer.

Details on the measurement of covariates

Potential confounders included demographics, education level, smoking history, comorbidities, medication use, dialysis access, and dialysis clearance. Participants' demographic factors (age, sex, and race), education level, smoking history, and medication use were self-reported. Comorbidities including diabetes mellitus, coronary artery disease, and hypertension were adjudicated by a committee of physicians. Dialysis clearance was assessed by single-pool Kt/V (spKt/V).

We also measured serum markers of mineral metabolism, circulating inhibitors of cardiovascular calcification, calciprotein particle size and transformation time, and C-reactive protein. All measurements were performed in the same serum samples as the metabolomics, except for serum

calcium, phosphorous, and intact parathyroid hormone (PTH) levels, which were averaged from 3-months of lab values collected before a dialysis session. C-terminal fibroblast growth factor (FGF23, CV=13%; Immutopics, San Clemente, CA, USA) and soluble klotho (CV=2.3%; Immuno-biological Laboratories, Takasaki, Japan) were measured using enzyme-linked immunosorbent assays (ELISA). For circulating inhibitors of cardiovascular calcification, osteoprotegerin² (CV=9%; Alpaco Diagnostics, Salem, NH, USA), dephosphorylated and uncarboxylated matrix glutamate (Gla) protein (dp-ucMGP)^{3,4} (CV=11%; VitaK BV, Maastricht, Netherlands), and fetuin-A⁵ (CV=18%; Epitope Diagnostics, San Diego, CA, USA) were measured using ELISA. The size of secondary calciprotein particle aggregates (CPP2; CV=13%) and the time of transformation from primary to secondary calciprotein particle (T_{50} ; CV=10%) were measured using dynamic light scattering as previously described.⁶ High sensitivity C-reactive protein (CV=7%; BNII Nephelometer; Siemens Healthcare, Germany) was measured using ELISA.

SUPPLEMENTAL TABLES

Table S1. Comparing baseline characteristics of study population with unselected participants

	Study population (n=99)	Diabetes (n=239)	No CAC score (n=291)	CAC score between 0-100 & non- diabetics (n=31)
Age, year (missing=200)	53 ± 16	56 ± 11*	56 ± 12	52 ± 11
Women, n (%) (missing=158)	38 (38)	98 (41)	132 (42)	16 (52)
African American, n (%) (missing=158)	71 (72)	169 (71)	223 (71)	23 (74)
Diabetes mellitus, n (%) (missing=158)	0 (0)	239 (100)*	239 (76)*	0 (0)
Hypertension, n (%) (missing=158)	99 (100)	239 (100)	316 (100%)	31 (100)
Coronary artery disease, n (%) (missing=158)	27 (27)	107 (45)*	127 (40)*	6 (19)

*p<0.05 compared to the study population. Age is provided as mean ± standard deviation and were tested using two-sample t-test. The rest are categorical variables, which are presented as absolute number with percentage and were tested with chi-square test.

Table S2. Summary of metabolites that differed between cases and controls

	Fold difference	Unadjusted p	Volcano plot	PLS-DA	Demographics-adjusted p
Arginine and proline metabolism					
Arginine	1.14	0.03		x	0.68
Ornithine	1.17	0.008		x	0.57
Succinic acid	1.44	0.005		x	0.21
Glycine and serine metabolism					
Betaine	1.30	0.0047	x	x	0.17
Tyrosine metabolism					
L-tyrosine	1.19	0.007		x	0.49
Methionine metabolism					
N-formyl-L-methionine	2.82	0.004	x	x	0.18
Amino acid degradation					
Methylguanidine	0.62	0.005		x	0.76
Fatty acid metabolism					
Suberic acid	1.25	0.004	x	x	0.16
Nonadecanoic acid	1.10	0.02		x	0.40
Bile acid synthesis					
Chenodeoxycholic acid	2.33	0.002	x	x	0.049
Deoxycholic acid	2.32	0.001	x	x	0.04
Glycolithocholic acid	2.44	0.004	x	x	0.04
Creatine phosphate pathway: muscle breakdown					
Creatinine	0.77	0.003	x	x	0.68
Glycolysis, glyoxylate and tricarboxylic acid cycle metabolism					
Citric acid	1.25	0.002	x	x	0.13
Food component					
Lumazine	1.22	0.002	x	x	0.49

Abbreviation: PLS-DA, partial least squares discriminant analysis

Using Mann-Whitney U test and a set threshold of fold difference (1.0) and raw p-value (0.005), we identified 9 metabolites that differed between cases and controls and showed them in the volcano plot. Using PLS-DA, we identified top 15 metabolites based on VIP scores. All 9 metabolites revealed by the volcano plot were also identified by the PLS-DA. After adjusting for demographics (age, sex, and race), only 3 bile acids remained associated with CAC status ($p < 0.05$).

Table S3. Tobit regression of log-transformed CAC score*

Metabolites[#]						
	Unadjusted		Demographics-adjusted		Fully adjusted	
	Coefficient (95% CI)	p	Coefficient (95% CI)	p	Coefficient (95% CI)	p
Chenodeoxycholic acid	0.27 (0.07 – 0.46)	0.01	0.15 (0.03 – 0.27)	0.02	0.13 (0.01 – 0.25)	0.03
Deoxycholic acid	0.28 (0.06 – 0.50)	0.01	0.16 (0.04 – 0.29)	0.01	0.14 (-0.001 – 0.28)	0.05
Glycolithocholic acid (per 0.01 unit)	0.10 (0.04 – 0.16)	0.01	0.05 (0.01 – 0.09)	0.01	0.01 (-0.05 – 0.06)	0.74
Pathways[#]						
	Unadjusted		Demographics-adjusted		Fully adjusted	
	Coefficient (95% CI)	p	Coefficient (95% CI)	p	Coefficient (95% CI)	p
Arginine/proline metabolism	0.91 (0.19 – 1.62)	0.01	0.61 (0.12 – 1.11)	0.02	0.58 (0.11 – 1.05)	0.02
Urea cycle	1.61 (0.89 – 2.34)	<0.001	0.65 (0.003 – 1.29)	0.049	0.65 (0.02 – 1.29)	0.04
Bile acid synthesis	-0.02 (-0.75 – 0.71)	0.96	0.11 (-0.38 – 0.61)	0.65	0.22 (-0.29 – 0.73)	0.40

*CAC score was log-transformed after adding 1 (i.e. $\ln(\text{CAC score} + 1)$). [#]per 1 unit higher in relative intensity unless otherwise specified for analyses of metabolites; per 1 unit higher in the first principal component score of each pathway for analyses of pathways.

Demographics included age, sex, and race. Full models were adjusted for demographics, coronary artery disease, use of calcium-based phosphate binder, renin-angiotensin-aldosterone system blockage and statin for all 3 bile acids and bile acid synthesis pathway, and were adjusted for demographics and statin use for arginine/proline metabolism and urea cycle pathways.

Table S4. Metabolized analyzed in arginine/proline metabolism, urea cycle and bile acid synthesis

Arginine and proline metabolism (m=15)		Urea Cycle (m=10)		Bile acid synthesis (m=12)	
Metabolites	HMDB ID	Metabolites	HMDB ID	Metabolites	HMDB ID
L-arginine*	0000517	L-arginine*	0000517	Glycine	0000123
Ornithine*	0000214	Ornithine*	0000214	Glycocholic acid	0000138
Citrulline*	0000904	L-glutamine*	0000641	Palmitic acid	0000220
L-aspartic acid	0000191	Citrulline*	0000904	Taurine	0000251
Fumaric acid	0000134	L-alanine	0000161	Chenodeoxycholic acid*	0000518
Oxalacetic acid	0000223	L-aspartic acid	0000191	Glycochenodeoxycholic acid	0000637
L-glutamic acid	0000148	Fumaric acid	0000134	Taurochenodeoxycholic acid	0000951
Succinic acid*	0000254	Oxalacetic acid	0000223	Deoxycholic acid*	0000626
4-hydroxy-L-proline*	0006055	Pyruvic acid	0000243	Glycodeoxycholic acid	0000631
S-adenosylmethionine	0001185	L-glutamic acid	0000148	Taurodeoxycholic acid	0000896
Flavin adenine dinucleotide	0001248			Glycolithocholic acid*	0000698
Glycine	0000123			Flavin adenine dinucleotide	0001248
L-proline	0000162				
Creatine	0000064				
S-adenosylhomocysteine	0000939				

*p<0.05, comparing cases with controls for each metabolite. Abbreviation: HMDB, human metabolome database identification

Table S5. Correlations of bile acid synthesis and arginine/proline metabolism pathways and their key metabolites with serum markers of mineral metabolism (Ca, Phos, Mg, PTH, FGF23, klotho), circulating inhibitors of calcification (osteoprotegerin, dp-ucMGP, fetuin-A, CPP2, T₅₀) and inflammation (CRP).

	Ca	Phos	Mg	PTH	FGF23	Klotho	Osteoprotegerin	Dp-ucMGP	Fetuin-A	CPP2	T50	CRP
Bile acid synthesis pathway	-0.14	0.13	0.03	0.17	0.24*	-0.09	0.33**	0.03	-0.08	0.17	-0.23*	-0.15
Glycocholic acid	-0.06	0.14	-0.03	0.06	0.25*	-0.03	0.2*	0.04	-0.01	0.1	-0.12	-0.17
Chenodeoxycholic acid	0.03	0.05	0.08	0.07	-0.12	-0.17	0.09	-0.11	0.07	-0.02	-0.05	-0.24*
Glychenodeoxycholic acid	-0.25*	0.08	-0.001	0.12	0.16	-0.06	0.29**	0.1	-0.02	0.11	-0.15	-0.16
Taurochenodeoxycholic acid	-0.14	0.01	0.02	0.13	0.24*	0.08	0.23*	0.08	-0.03	0.11	-0.16	-0.14
Deoxycholic acid	0.02	0.06	0.08	0.09	-0.11	-0.17	0.08	-0.09	0.07	-0.02	-0.03	-0.22*
Glycodeoxycholic acid	-0.25*	0.09	0.01	0.11	0.16	-0.06	0.28**	0.1	-0.02	0.11	-0.14	-0.16
Taurodeoxycholic acid	-0.19	0.002	0.09	0.15	0.2	0.06	0.22*	0.08	-0.08	0.14	-0.15	-0.17
Glycolithocholic acid	0.11	0.12	0.16	0.23*	-0.03	-0.08	0.03	-0.14	0.15	-0.05	-0.02	-0.23*
Argine/proline metabolism pathway	-0.17	0.22*	0.17	0.03	0.27*	-0.0002	0.33**	0.22*	0.1	-0.04	-0.03	-0.15
L-arginine	0.08	0.1	0.05	0.01	-0.06	-0.15	0.05	0.07	-0.04	-0.01	0.001	0.23*
Ornithine	-0.19	0.06	0.01	-0.07	0.02	-0.26*	0.44**	0.07	-0.1	0.09	-0.03	-0.18
Citrulline	0.09	0.24*	0.31**	0.01	0.21*	-0.13	0.25*	0.05	0.08	-0.001	0.06	-0.13
Succinic acid	0.03	-0.07	-0.1	-0.1	0.06	0.04	0.37**	0.23*	0.09	-0.12	0.22*	-0.15
4-hydroxy-L-proline	0.01	-0.04	0.05	-0.05	0.05	-0.04	0.09	-0.06	-0.07	0.14	0.1	-0.03
L-glutamic acid	-0.11	-0.03	0.06	0.08	-0.05	-0.02	-0.08	0.03	0.25*	-0.34**	0.12	-0.09
L-proline	-0.14	0.28*	0.22*	0.13	0.24*	0.002	-0.03	0.12	0.2*	-0.02	-0.05	-0.12

Spearman's rank correlations were used and presented as Rho in the table. The first principal component scores were used to represent the pathways. *p<0.05; **p<0.005. Abbreviations: Ca, calcium; Phos, phosphorous; Mg, magnesium; PTH, intact parathyroid hormone; FGF-23, fibroblast growth factor-23; klotho, soluble klotho; dp-ucMGP, Dephosphorylated and uncarboxylated matrix Gla protein; CPP2, secondary calciprotein particle; T₅₀, half maximal transformation of primary to secondary calciprotein particle; CRP, C-reactive protein.

SUPPLEMENTAL FIGURES

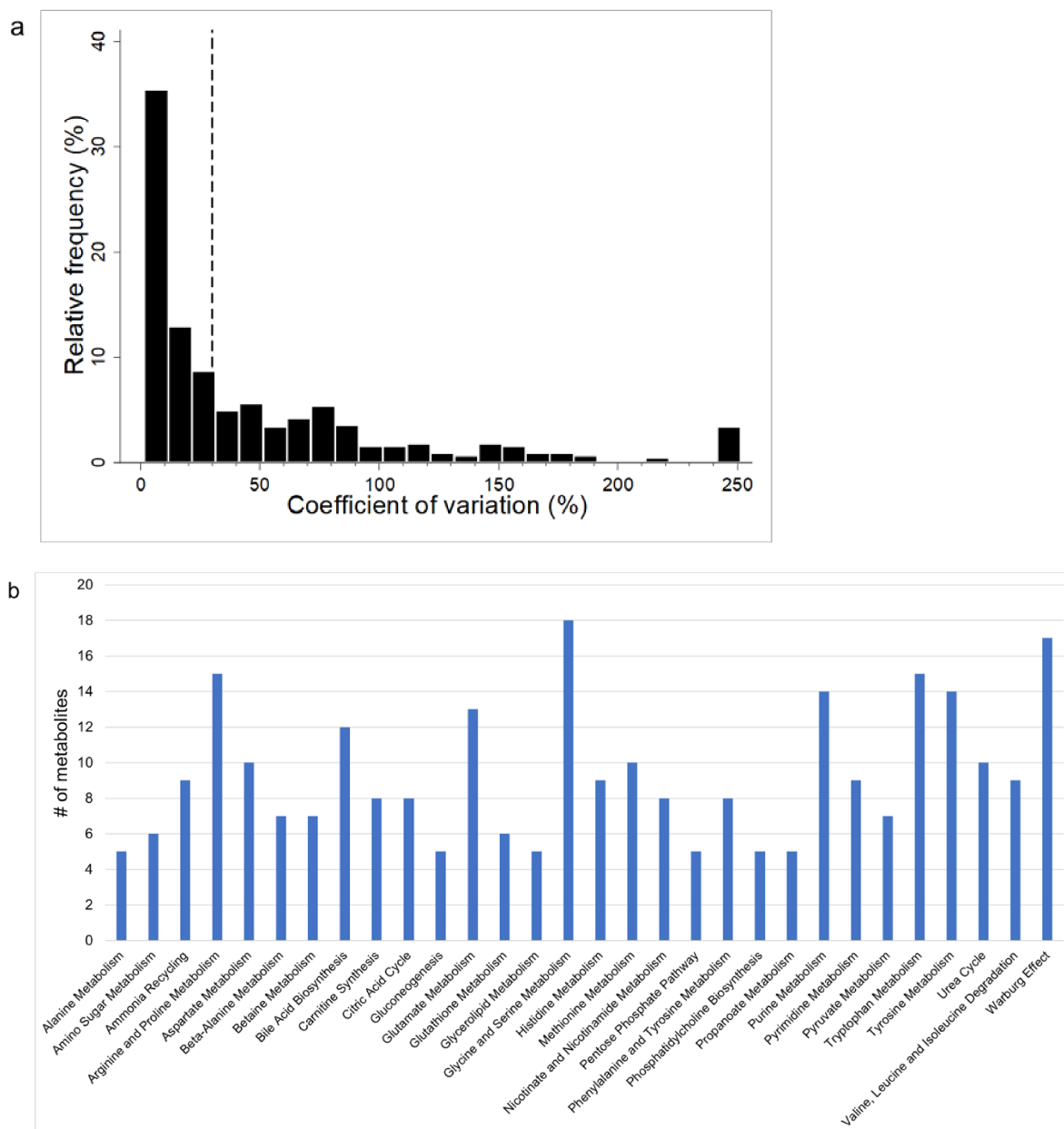


Figure S1. a) Distribution of coefficients of variation (CVs) for all metabolites measured (m=452). Reference line (dash line) is at CV of 30%. Approximately 55% of metabolites (m=247) had CV <30%, and were analyzed. b) Pathways and number of metabolites (m=247) analyzed. Pathways were identified using the Small Molecule Pathway Database. Only pathways that contains at least 5 metabolites analyzed are shown. Some metabolites appear in multiple pathways. The pathways that have the most metabolites measured and analyzed included glycine/serine metabolism, Warburg effect, arginine/proline metabolism, and tryptophan metabolism.

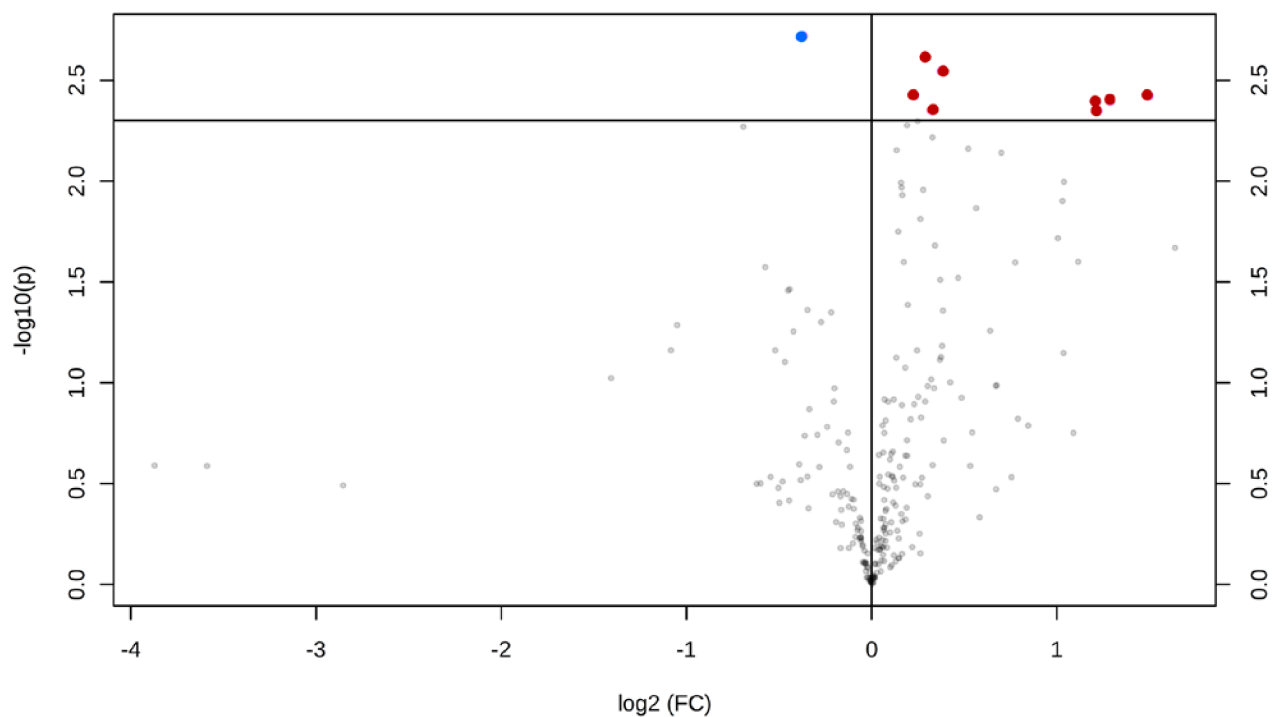


Figure S2. Volcano plot identified metabolites that differed between cases and controls. Compared to controls, cases had 8 metabolites that were higher in relative intensity (red dots) and 1 metabolite that was lower in relative intensity (blue dot). These metabolites and their fold differences are listed in Table S1. Mann-Whitney U tests were used with p-value threshold of <0.005 and a fold difference threshold of 1.0 to obtain approximately 10 metabolites.

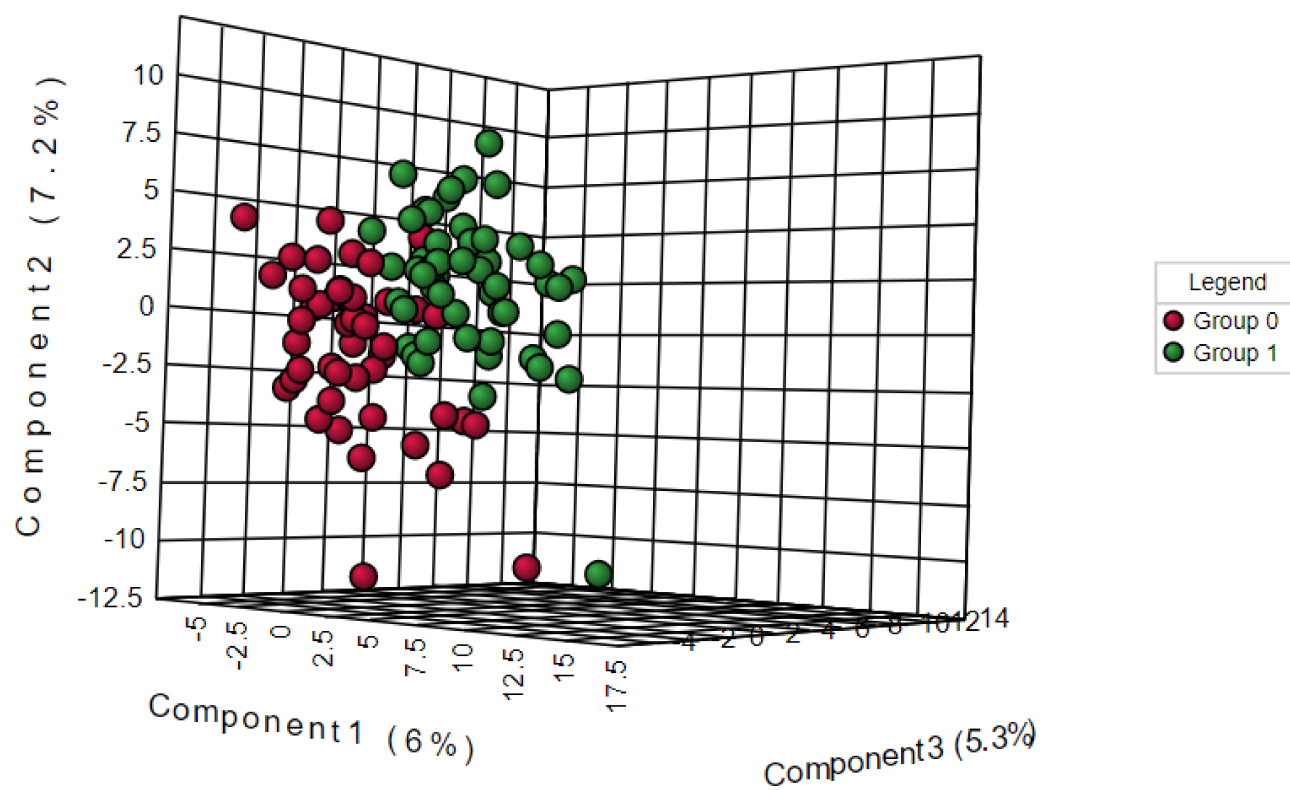


Figure S3. Synchronized 3-dimensional plot of partial least squares-discriminant analyses. Cases are in green and controls are in red.

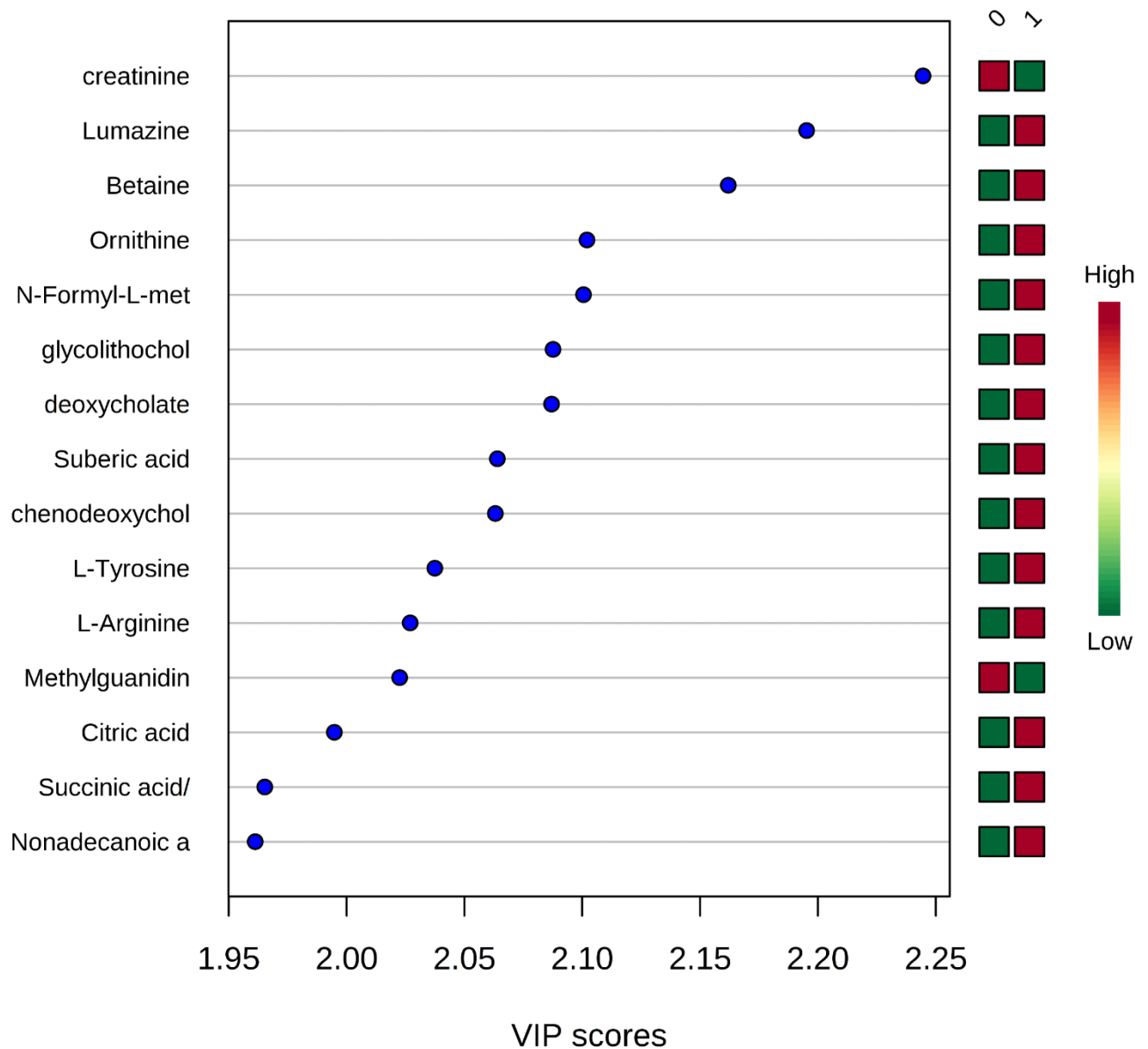


Figure S4. Top 15 serum metabolites listed by their Variable Importance in Projection scores (using the first principal component) using partial least squares discriminant analyses. The colored boxes on the right indicate the relative concentrations of the corresponding metabolites in cases (labeled as 1) and controls (labeled as 0). Red indicates high relative concentration and green indicates low relative concentration. Abbreviation: VIP, variable importance in project.

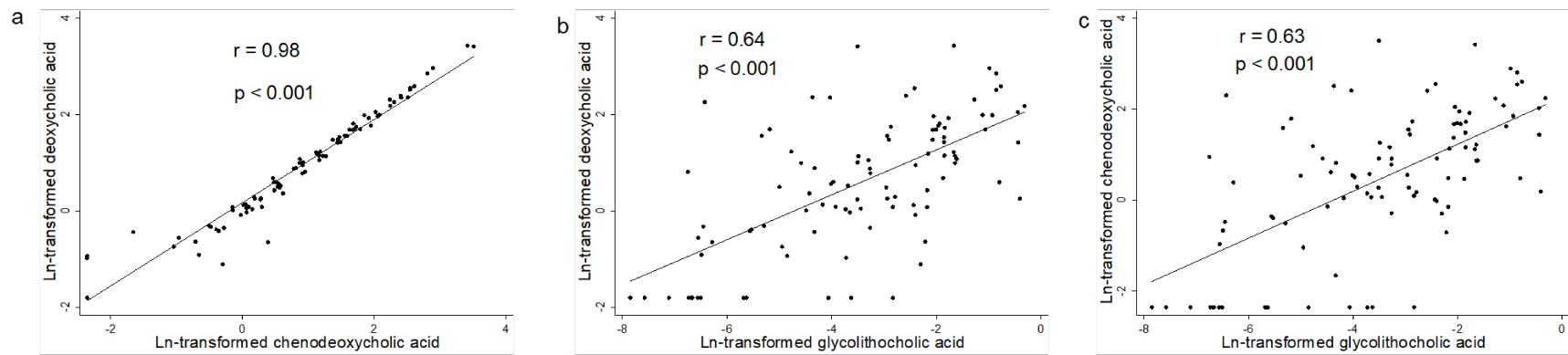


Figure S5. Correlation among 3 bile acids. Bile acids positively correlated with each other. Pearson Correlation was used after log-transformation of bile acid levels.

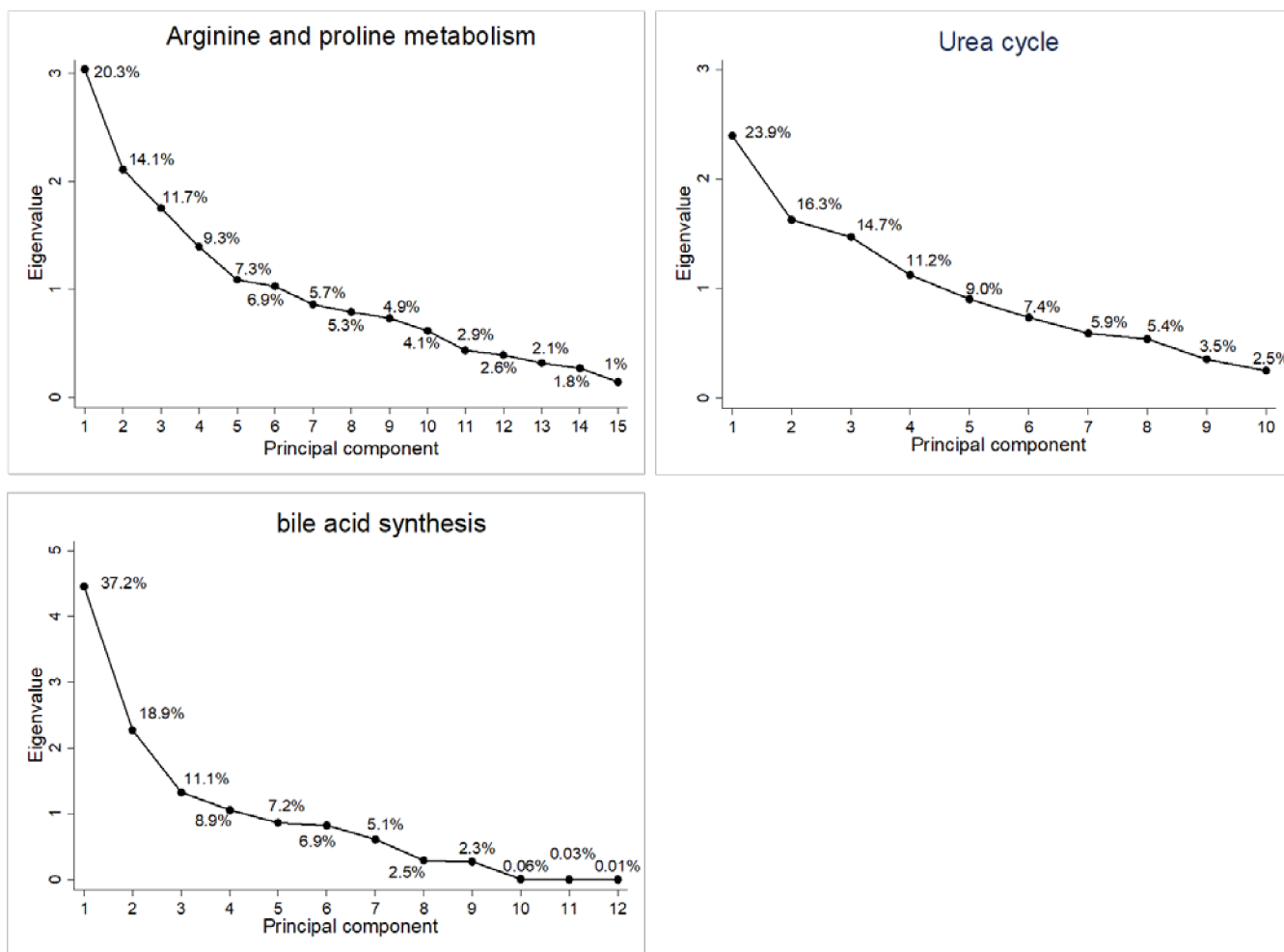
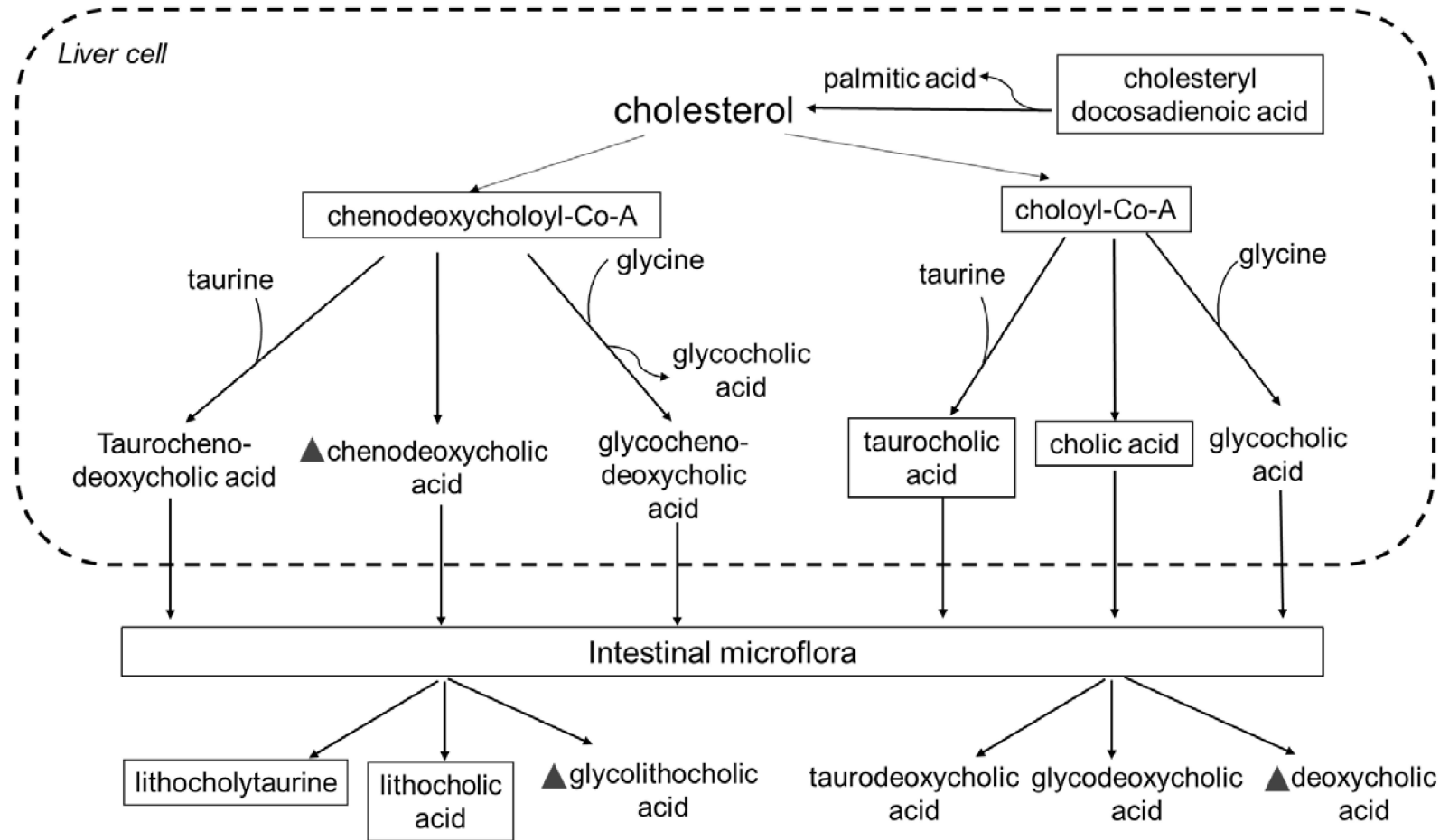


Figure S6. Scree plots of arginine/proline metabolism (m=15), urea cycle (m=10) and bile acid synthesis (m=12). Principal component analysis was used to generate principal component scores of arginine/proline metabolism, urea cycle and bile acid synthesis. X-axis is the principal component number; y-axis is the eigenvalue. Variance explained by individual principal component is annotated on each curve.

a.



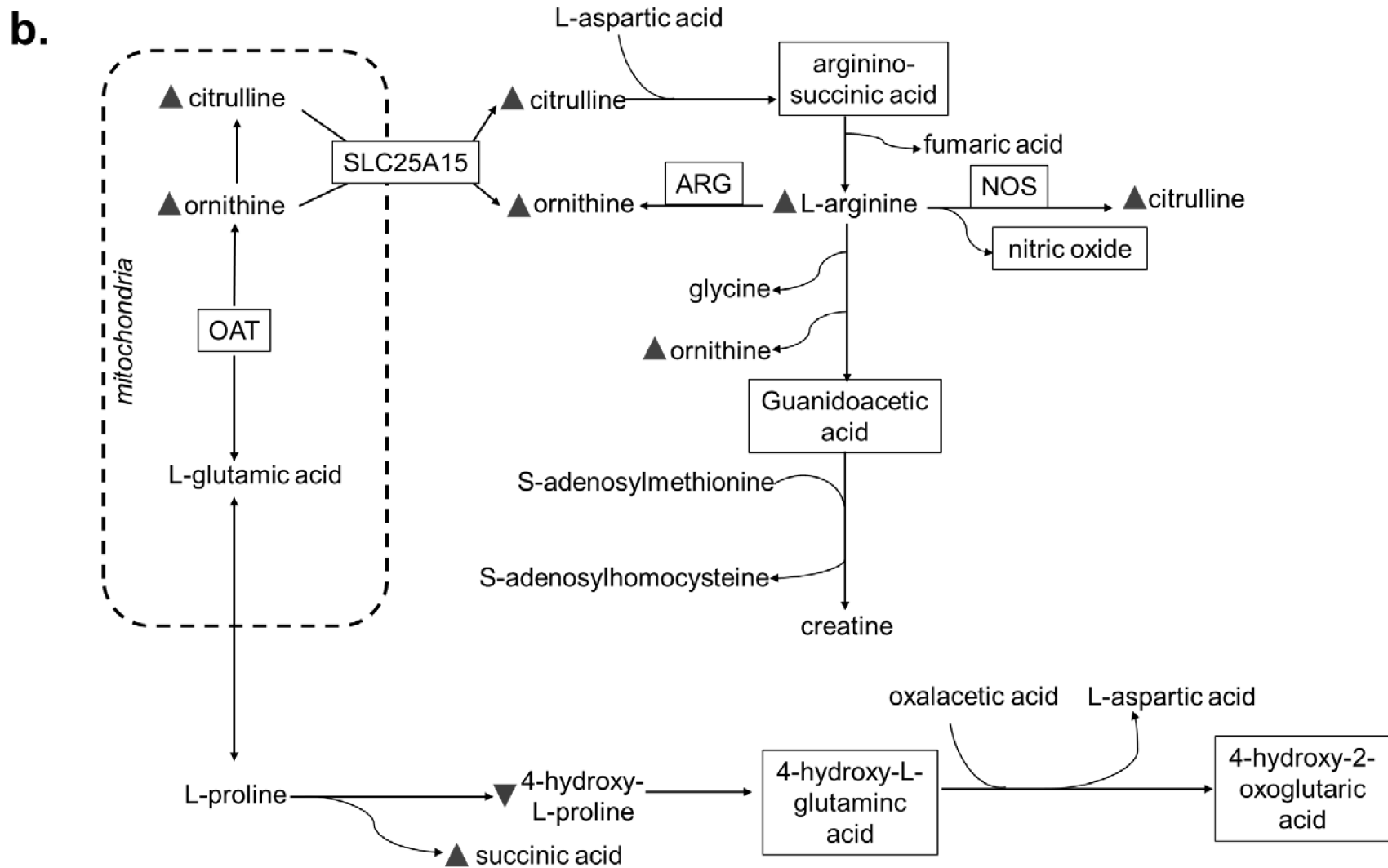


Figure S7. Bile acid synthesis and intracellular arginine and proline metabolism, simplified version (based on the Small Molecule Pathway Database). (a) Bile acid synthesis. Chenodeoxycholic acid is a primary bile acid as it is directly synthesized from cholesterol in liver. After being synthesized, chenodeoxycholic acid is then secreted into bile and intestinal tract, where it is converted by intestinal bacteria to lithocholic acid, which is a secondary bile acid. To increase its solubility and decrease toxicity, lithocholic acid is conjugated with glycine to form glycolithocholic acid. Cholic acid is another primary bile acid. In the intestinal tract, cholic acid is

converted to deoxycholic acid, a secondary bile acid. We found that higher levels of chenodeoxycholic, deoxycholic, and glycolithocholic acids were associated with the presence and severity of CAC. (b) Intracellular arginine and proline metabolism. Arginine/proline metabolism is one of the central pathways for the biosynthesis of arginine and proline from glutamine. We found that arginine/proline metabolism was independently associated with CAC status. Compared to controls, cases had higher levels of L-arginine, ornithine, citrulline, and succinic acid and lower levels of 4-hydroxy-L-proline ($p < 0.05$). Assuming intracellular metabolism of these amino acids mirrors their extracellular metabolism and serum levels, their directionality seems to suggest that there was an increased synthesis of L-arginine and decreased metabolism of proline in participants with CAC.

Note: Metabolites that either were not measured or had a $CV \geq 30\%$ are in boxes. The rest are metabolites analyzed. Total cholesterol level was measured separately in fasting blood samples on a non-HD day. Arrowheads ($\blacktriangle \blacktriangledown$) indicate the directionality when comparing the cases with controls ($p < 0.05$). Abbreviations: OAT, ornithine aminotransferase; SLC25A15, mitochondrial ornithine transporter 1; ARG, arginase; NOS, nitric oxide synthase.

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Supplemental Material

Title: Identification of Novel Biomarkers and Pathways for Coronary Artery Calcification in Non-diabetic Patients on Hemodialysis Using Metabolomic Profiling

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Supplemental Figures

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Figure S7. Bile acid synthesis and intracellular arginine and proline metabolism, simplified version.

References

SUPPLEMENTAL METHODS

Selection of study population

In the PACE cohort (n=568), diabetes status and blood samples were not available in 158 participants because these participants did not complete the study visit. Every participant in the PACE cohort had hypertension. In current study, we compared the serum metabolites of non-diabetics with a CAC score >100 (case) with non-diabetics with a CAC score of 0 (control). Compared to the study population, participants with diabetes were older (56 ± 11 vs. 53 ± 16 years) and more likely to have CAD (45% vs. 27%; Table S1). Participants without available CAC scores (76% had diabetes) were more likely to have CAD (40%) compared to the study population. Non-diabetic participants with a CAC score between 0-100 had similar demographic characteristics with the study population.

Details on CAC measurement

CAC was measured at the baseline visit by computed tomography (Toshiba Aquilon One, Japan), acquired prospectively in synchrony with the electrocardiogram at 70-80% RR interval, near the end of diastole and before atrial contraction.¹ Coronary calcium was quantified by Agatston score. CAC scores were assessed and then validated by a second reviewer.

Details on the measurement of covariates

Potential confounders included demographics, education level, smoking history, comorbidities, medication use, dialysis access, and dialysis clearance. Participants' demographic factors (age, sex, and race), education level, smoking history, and medication use were self-reported. Comorbidities including diabetes mellitus, coronary artery disease, and hypertension were adjudicated by a committee of physicians. Dialysis clearance was assessed by single-pool Kt/V (spKt/V).

We also measured serum markers of mineral metabolism, circulating inhibitors of cardiovascular calcification, calciprotein particle size and transformation time, and C-reactive protein. All measurements were performed in the same serum samples as the metabolomics, except for serum

calcium, phosphorous, and intact parathyroid hormone (PTH) levels, which were averaged from 3-months of lab values collected before a dialysis session. C-terminal fibroblast growth factor (FGF23, CV=13%; Immutopics, San Clemente, CA, USA) and soluble klotho (CV=2.3%; Immuno-biological Laboratories, Takasaki, Japan) were measured using enzyme-linked immunosorbent assays (ELISA). For circulating inhibitors of cardiovascular calcification, osteoprotegerin² (CV=9%; Alpaco Diagnostics, Salem, NH, USA), dephosphorylated and uncarboxylated matrix glutamate (Gla) protein (dp-ucMGP)^{3,4} (CV=11%; VitaK BV, Maastricht, Netherlands), and fetuin-A⁵ (CV=18%; Epitope Diagnostics, San Diego, CA, USA) were measured using ELISA. The size of secondary calciprotein particle aggregates (CPP2; CV=13%) and the time of transformation from primary to secondary calciprotein particle (T_{50} ; CV=10%) were measured using dynamic light scattering as previously described.⁶ High sensitivity C-reactive protein (CV=7%; BNII Nephelometer; Siemens Healthcare, Germany) was measured using ELISA.

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Table S1. Comparing baseline characteristics of study population with unselected participants

	Study population (n=99)	Diabetes (n=239)	No CAC score (n=291)	CAC score between 0-100 & non- diabetics (n=31)
Age, year (missing=200)	53 ± 16	56 ± 11*	56 ± 12	52 ± 11
Women, n (%) (missing=158)	38 (38)	98 (41)	132 (42)	16 (52)
African American, n (%) (missing=158)	71 (72)	169 (71)	223 (71)	23 (74)
Diabetes mellitus, n (%) (missing=158)	0 (0)	239 (100)*	239 (76)*	0 (0)
Hypertension, n (%) (missing=158)	99 (100)	239 (100)	316 (100%)	31 (100)
Coronary artery disease, n (%) (missing=158)	27 (27)	107 (45)*	127 (40)*	6 (19)

*p<0.05 compared to the study population. Age is provided as mean ± standard deviation and were tested using two-sample t-test. The rest are categorical variables, which are presented as absolute number with percentage and were tested with chi-square test.

Table S2. Summary of metabolites that differed between cases and controls

	Fold difference	Unadjusted p	Volcano plot	PLS-DA	Demographics-adjusted p
Arginine and proline metabolism					
Arginine	1.14	0.03		x	0.68
Ornithine	1.17	0.008		x	0.57
Succinic acid	1.44	0.005		x	0.21
Glycine and serine metabolism					
Betaine	1.30	0.0047	x	x	0.17
Tyrosine metabolism					
L-tyrosine	1.19	0.007		x	0.49
Methionine metabolism					
N-formyl-L-methionine	2.82	0.004	x	x	0.18
Amino acid degradation					
Methylguanidine	0.62	0.005		x	0.76
Fatty acid metabolism					
Suberic acid	1.25	0.004	x	x	0.16
Nonadecanoic acid	1.10	0.02		x	0.40
Bile acid synthesis					
Chenodeoxycholic acid	2.33	0.002	x	x	0.049
Deoxycholic acid	2.32	0.001	x	x	0.04
Glycolithocholic acid	2.44	0.004	x	x	0.04
Creatine phosphate pathway: muscle breakdown					
Creatinine	0.77	0.003	x	x	0.68
Glycolysis, glyoxylate and tricarboxylic acid cycle metabolism					
Citric acid	1.25	0.002	x	x	0.13
Food component					
Lumazine	1.22	0.002	x	x	0.49

Abbreviation: PLS-DA, partial least squares discriminant analysis

Using Mann-Whitney U test and a set threshold of fold difference (1.0) and raw p-value (0.005), we identified 9 metabolites that differed between cases and controls and showed them in the volcano plot. Using PLS-DA, we identified top 15 metabolites based on VIP scores. All 9 metabolites revealed by the volcano plot were also identified by the PLS-DA. After adjusting for demographics (age, sex, and race), only 3 bile acids remained associated with CAC status ($p < 0.05$).

Table S3. Tobit regression of log-transformed CAC score*

Metabolites[#]						
	Unadjusted		Demographics-adjusted		Fully adjusted	
	Coefficient (95% CI)	p	Coefficient (95% CI)	p	Coefficient (95% CI)	p
Chenodeoxycholic acid	0.27 (0.07 – 0.46)	0.01	0.15 (0.03 – 0.27)	0.02	0.13 (0.01 – 0.25)	0.03
Deoxycholic acid	0.28 (0.06 – 0.50)	0.01	0.16 (0.04 – 0.29)	0.01	0.14 (-0.001 – 0.28)	0.05
Glycolithocholic acid (per 0.01 unit)	0.10 (0.04 – 0.16)	0.01	0.05 (0.01 – 0.09)	0.01	0.01 (-0.05 – 0.06)	0.74
Pathways[#]						
	Unadjusted		Demographics-adjusted		Fully adjusted	
	Coefficient (95% CI)	p	Coefficient (95% CI)	p	Coefficient (95% CI)	p
Arginine/proline metabolism	0.91 (0.19 – 1.62)	0.01	0.61 (0.12 – 1.11)	0.02	0.58 (0.11 – 1.05)	0.02
Urea cycle	1.61 (0.89 – 2.34)	<0.001	0.65 (0.003 – 1.29)	0.049	0.65 (0.02 – 1.29)	0.04
Bile acid synthesis	-0.02 (-0.75 – 0.71)	0.96	0.11 (-0.38 – 0.61)	0.65	0.22 (-0.29 – 0.73)	0.40

*CAC score was log-transformed after adding 1 (i.e. $\ln(\text{CAC score} + 1)$). [#]per 1 unit higher in relative intensity unless otherwise specified for analyses of metabolites; per 1 unit higher in the first principal component score of each pathway for analyses of pathways.

Demographics included age, sex, and race. Full models were adjusted for demographics, coronary artery disease, use of calcium-based phosphate binder, renin-angiotensin-aldosterone system blockage and statin for all 3 bile acids and bile acid synthesis pathway, and were adjusted for demographics and statin use for arginine/proline metabolism and urea cycle pathways.

Table S4. Metabolized analyzed in arginine/proline metabolism, urea cycle and bile acid synthesis

Arginine and proline metabolism (m=15)		Urea Cycle (m=10)		Bile acid synthesis (m=12)	
Metabolites	HMDB ID	Metabolites	HMDB ID	Metabolites	HMDB ID
L-arginine*	0000517	L-arginine*	0000517	Glycine	0000123
Ornithine*	0000214	Ornithine*	0000214	Glycocholic acid	0000138
Citrulline*	0000904	L-glutamine*	0000641	Palmitic acid	0000220
L-aspartic acid	0000191	Citrulline*	0000904	Taurine	0000251
Fumaric acid	0000134	L-alanine	0000161	Chenodeoxycholic acid*	0000518
Oxalacetic acid	0000223	L-aspartic acid	0000191	Glycochenodeoxycholic acid	0000637
L-glutamic acid	0000148	Fumaric acid	0000134	Taurochenodeoxycholic acid	0000951
Succinic acid*	0000254	Oxalacetic acid	0000223	Deoxycholic acid*	0000626
4-hydroxy-L-proline*	0006055	Pyruvic acid	0000243	Glycodeoxycholic acid	0000631
S-adenosylmethionine	0001185	L-glutamic acid	0000148	Taurodeoxycholic acid	0000896
Flavin adenine dinucleotide	0001248			Glycolithocholic acid*	0000698
Glycine	0000123			Flavin adenine dinucleotide	0001248
L-proline	0000162				
Creatine	0000064				
S-adenosylhomocysteine	0000939				

*p<0.05, comparing cases with controls for each metabolite. Abbreviation: HMDB, human metabolome database identification

Table S5. Correlations of bile acid synthesis and arginine/proline metabolism pathways and their key metabolites with serum markers of mineral metabolism (Ca, Phos, Mg, PTH, FGF23, klotho), circulating inhibitors of calcification (osteoprotegerin, dp-ucMGP, fetuin-A, CPP2, T₅₀) and inflammation (CRP).

	Ca	Phos	Mg	PTH	FGF23	Klotho	Osteoprotegerin	Dp-ucMGP	Fetuin-A	CPP2	T50	CRP
Bile acid synthesis pathway	-0.14	0.13	0.03	0.17	0.24*	-0.09	0.33**	0.03	-0.08	0.17	-0.23*	-0.15
Glycocholic acid	-0.06	0.14	-0.03	0.06	0.25*	-0.03	0.2*	0.04	-0.01	0.1	-0.12	-0.17
Chenodeoxycholic acid	0.03	0.05	0.08	0.07	-0.12	-0.17	0.09	-0.11	0.07	-0.02	-0.05	-0.24*
Glychenodeoxycholic acid	-0.25*	0.08	-0.001	0.12	0.16	-0.06	0.29**	0.1	-0.02	0.11	-0.15	-0.16
Taurochenodeoxycholic acid	-0.14	0.01	0.02	0.13	0.24*	0.08	0.23*	0.08	-0.03	0.11	-0.16	-0.14
Deoxycholic acid	0.02	0.06	0.08	0.09	-0.11	-0.17	0.08	-0.09	0.07	-0.02	-0.03	-0.22*
Glycodeoxycholic acid	-0.25*	0.09	0.01	0.11	0.16	-0.06	0.28**	0.1	-0.02	0.11	-0.14	-0.16
Taurodeoxycholic acid	-0.19	0.002	0.09	0.15	0.2	0.06	0.22*	0.08	-0.08	0.14	-0.15	-0.17
Glycolithocholic acid	0.11	0.12	0.16	0.23*	-0.03	-0.08	0.03	-0.14	0.15	-0.05	-0.02	-0.23*
Argine/proline metabolism pathway	-0.17	0.22*	0.17	0.03	0.27*	-0.0002	0.33**	0.22*	0.1	-0.04	-0.03	-0.15
L-arginine	0.08	0.1	0.05	0.01	-0.06	-0.15	0.05	0.07	-0.04	-0.01	0.001	0.23*
Ornithine	-0.19	0.06	0.01	-0.07	0.02	-0.26*	0.44**	0.07	-0.1	0.09	-0.03	-0.18
Citrulline	0.09	0.24*	0.31**	0.01	0.21*	-0.13	0.25*	0.05	0.08	-0.001	0.06	-0.13
Succinic acid	0.03	-0.07	-0.1	-0.1	0.06	0.04	0.37**	0.23*	0.09	-0.12	0.22*	-0.15
4-hydroxy-L-proline	0.01	-0.04	0.05	-0.05	0.05	-0.04	0.09	-0.06	-0.07	0.14	0.1	-0.03
L-glutamic acid	-0.11	-0.03	0.06	0.08	-0.05	-0.02	-0.08	0.03	0.25*	-0.34**	0.12	-0.09
L-proline	-0.14	0.28*	0.22*	0.13	0.24*	0.002	-0.03	0.12	0.2*	-0.02	-0.05	-0.12

Spearman's rank correlations were used and presented as Rho in the table. The first principal component scores were used to represent the pathways. *p<0.05; **p<0.005. Abbreviations: Ca, calcium; Phos, phosphorous; Mg, magnesium; PTH, intact parathyroid hormone; FGF-23, fibroblast growth factor-23; klotho, soluble klotho; dp-ucMGP, Dephosphorylated and uncarboxylated matrix Gla protein; CPP2, secondary calciprotein particle; T₅₀, half maximal transformation of primary to secondary calciprotein particle; CRP, C-reactive protein.

SUPPLEMENTAL FIGURES

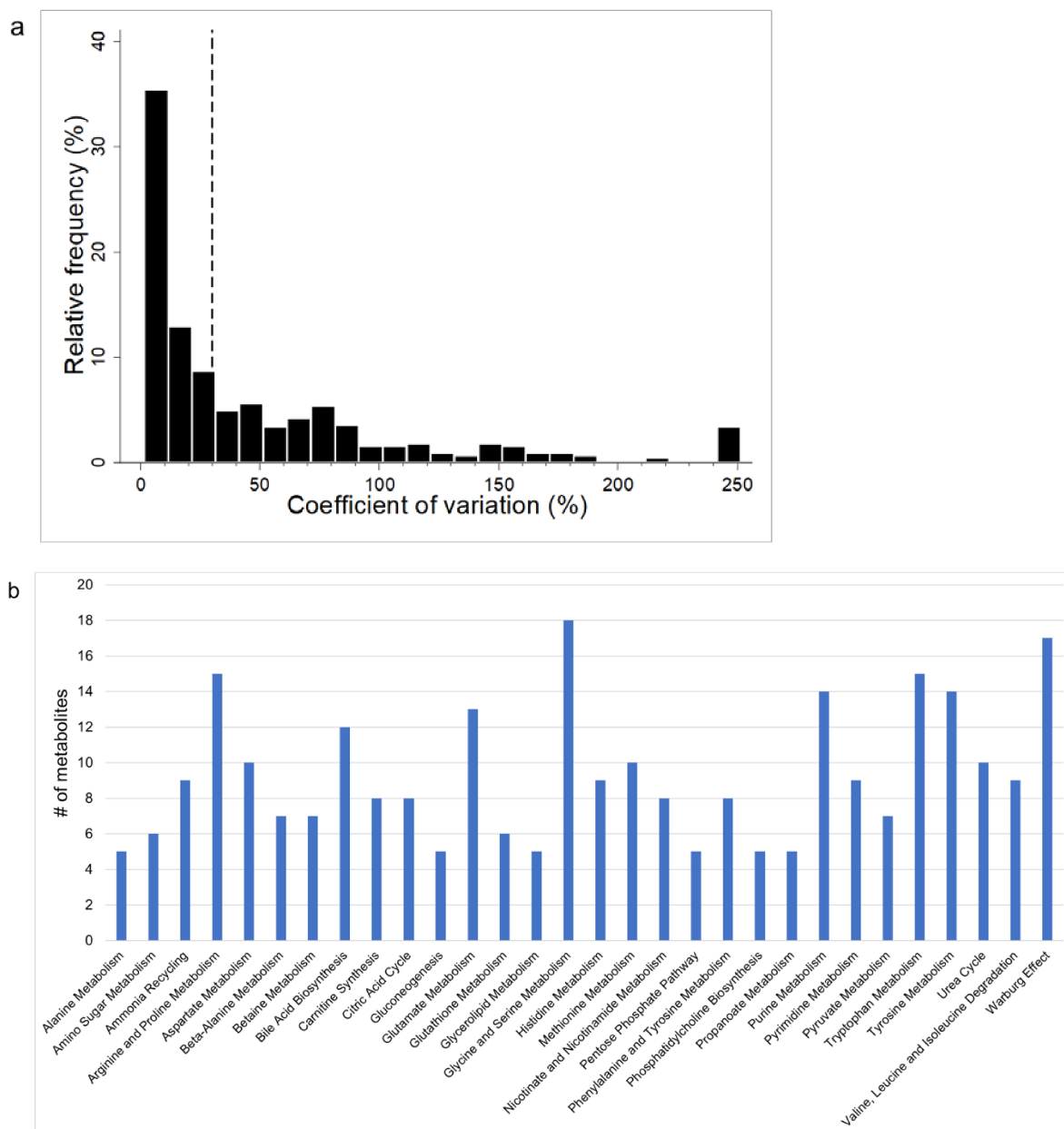


Figure S1. a) Distribution of coefficients of variation (CVs) for all metabolites measured (m=452). Reference line (dash line) is at CV of 30%. Approximately 55% of metabolites (m=247) had CV <30%, and were analyzed. b) Pathways and number of metabolites (m=247) analyzed. Pathways were identified using the Small Molecule Pathway Database. Only pathways that contains at least 5 metabolites analyzed are shown. Some metabolites appear in multiple pathways. The pathways that have the most metabolites measured and analyzed included glycine/serine metabolism, Warburg effect, arginine/proline metabolism, and tryptophan metabolism.

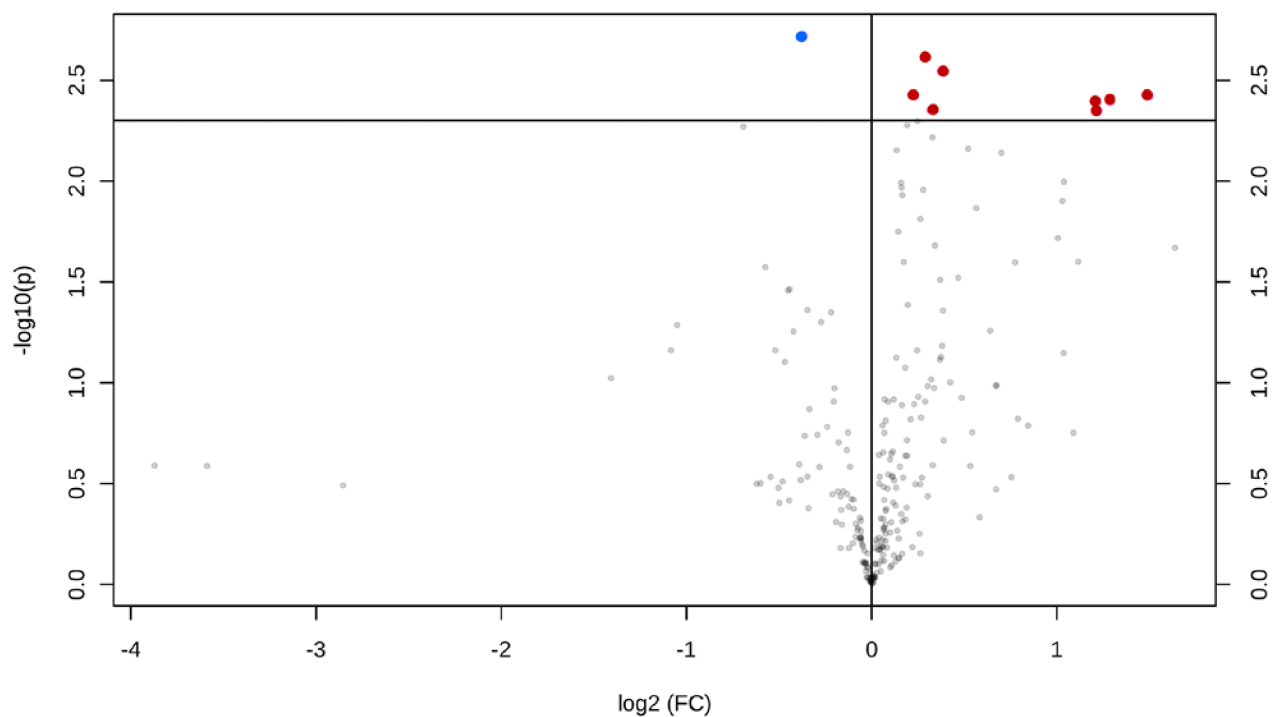


Figure S2. Volcano plot identified metabolites that differed between cases and controls. Compared to controls, cases had 8 metabolites that were higher in relative intensity (red dots) and 1 metabolite that was lower in relative intensity (blue dot). These metabolites and their fold differences are listed in Table S1. Mann-Whitney U tests were used with p-value threshold of <0.005 and a fold difference threshold of 1.0 to obtain approximately 10 metabolites.

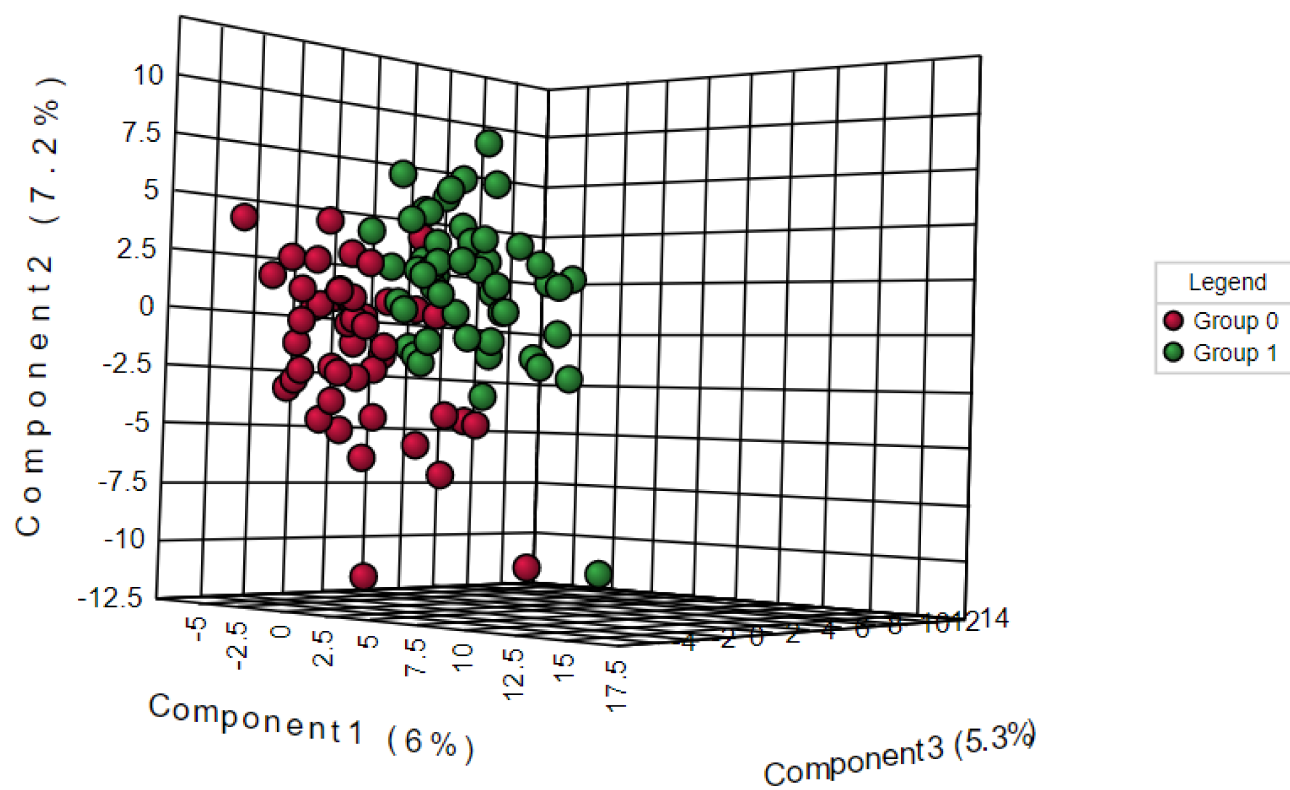


Figure S3. Synchronized 3-dimensional plot of partial least squares-discriminant analyses. Cases are in green and controls are in red.

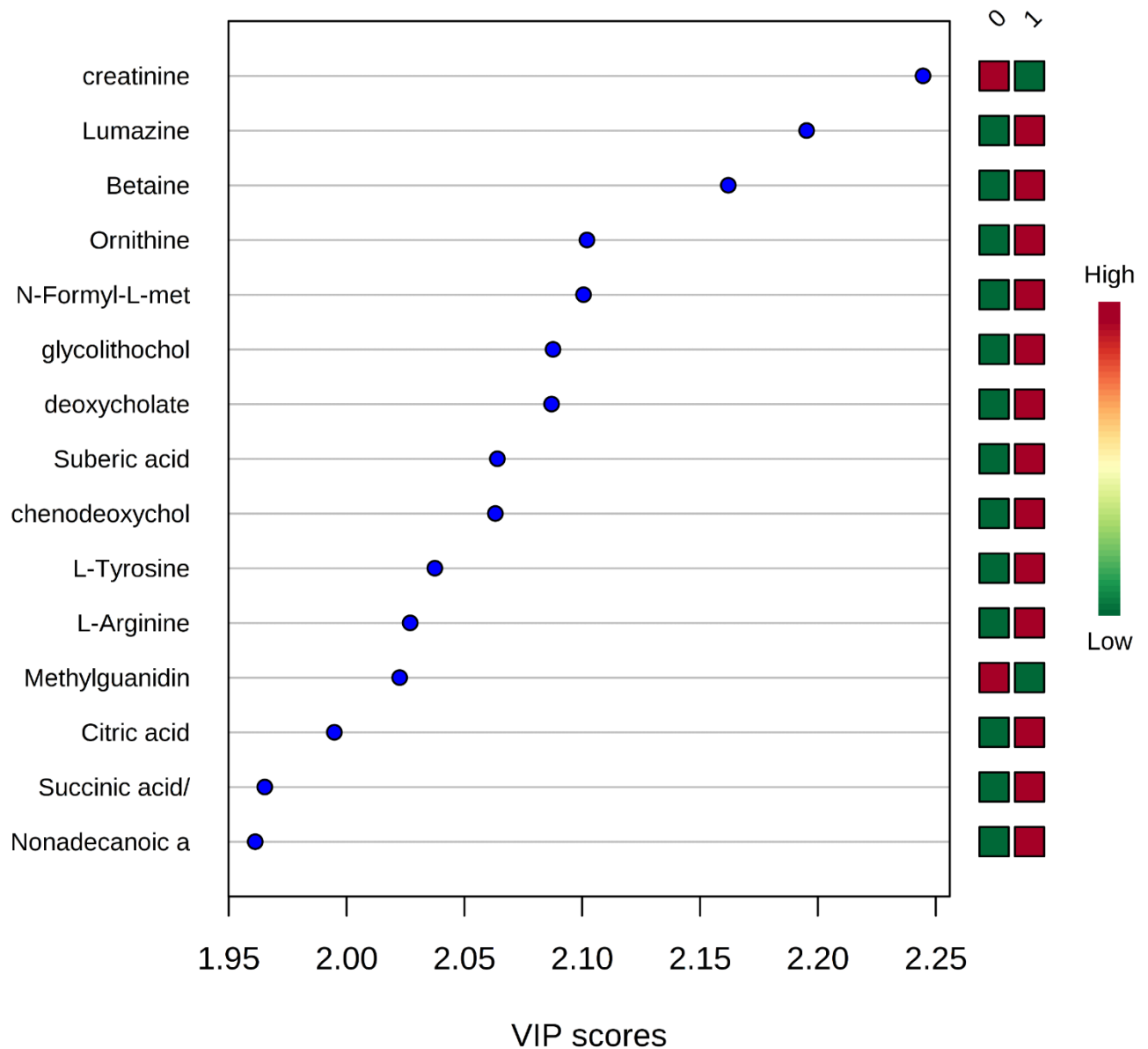


Figure S4. Top 15 serum metabolites listed by their Variable Importance in Projection scores (using the first principal component) using partial least squares discriminant analyses. The colored boxes on the right indicate the relative concentrations of the corresponding metabolites in cases (labeled as 1) and controls (labeled as 0). Red indicates high relative concentration and green indicates low relative concentration. Abbreviation: VIP, variable importance in project.

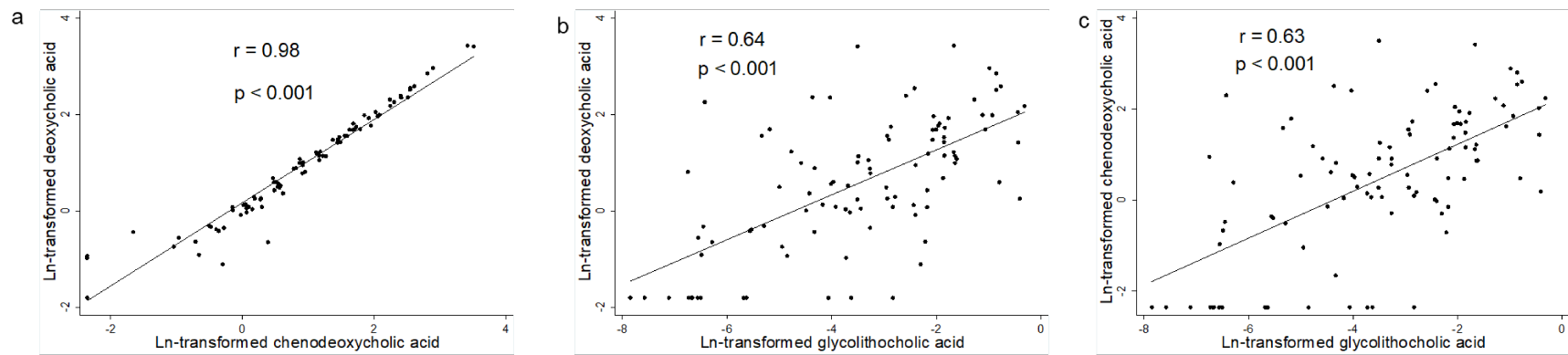


Figure S5. Correlation among 3 bile acids. Bile acids positively correlated with each other. Pearson Correlation was used after log-transformation of bile acid levels.

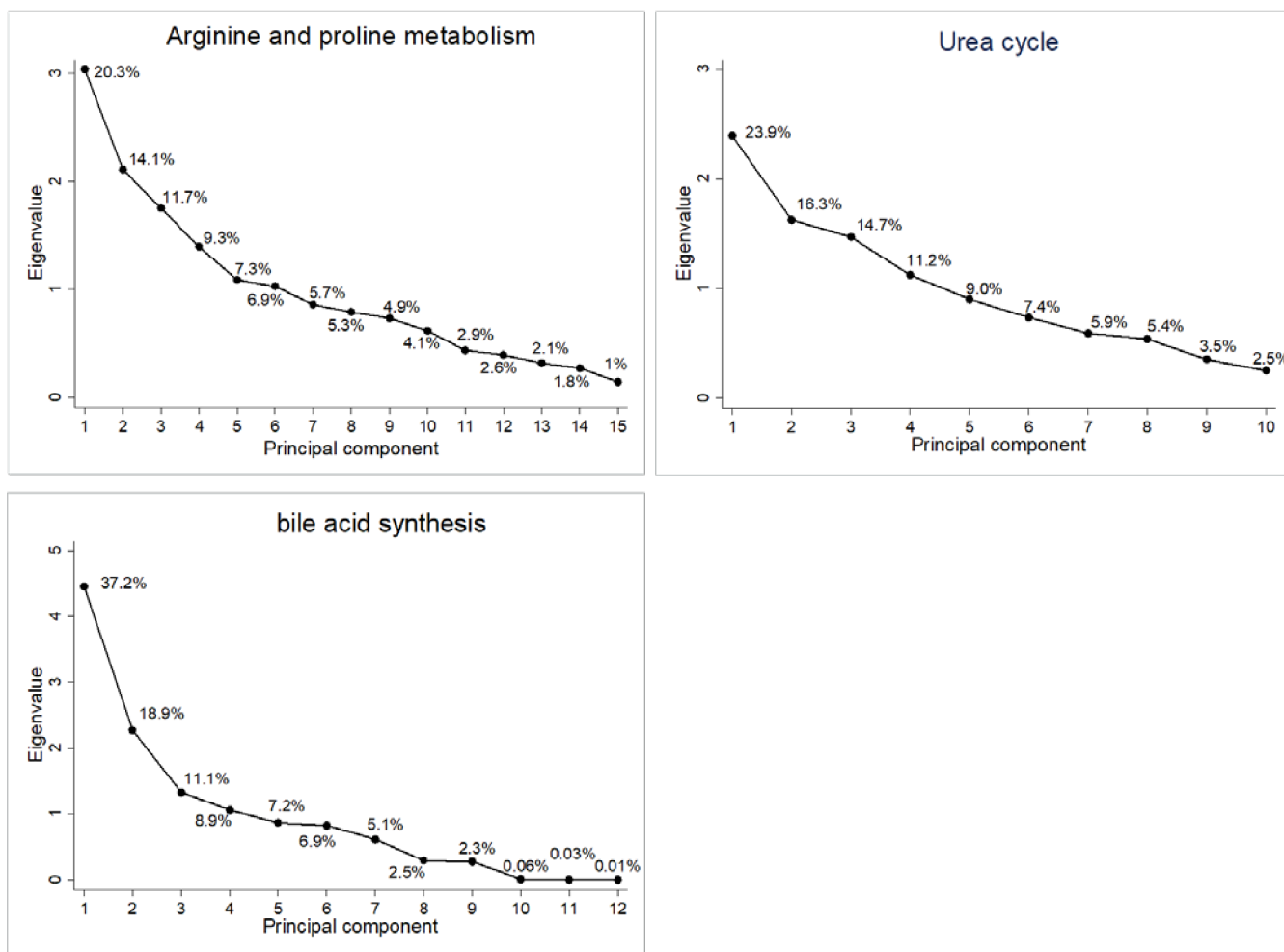
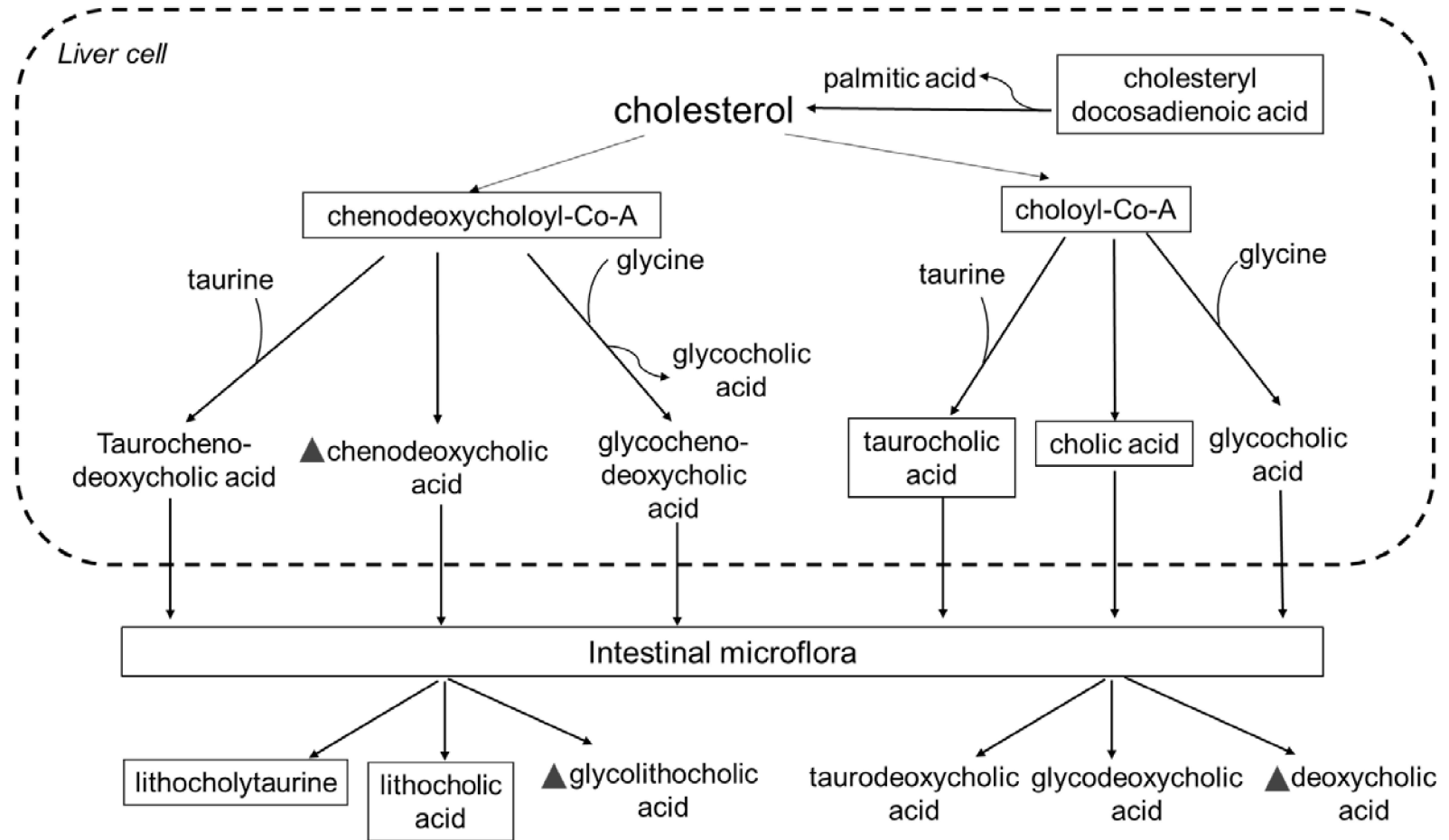


Figure S6. Scree plots of arginine/proline metabolism (m=15), urea cycle (m=10) and bile acid synthesis (m=12). Principal component analysis was used to generate principal component scores of arginine/proline metabolism, urea cycle and bile acid synthesis. X-axis is the principal component number; y-axis is the eigenvalue. Variance explained by individual principal component is annotated on each curve.

a.



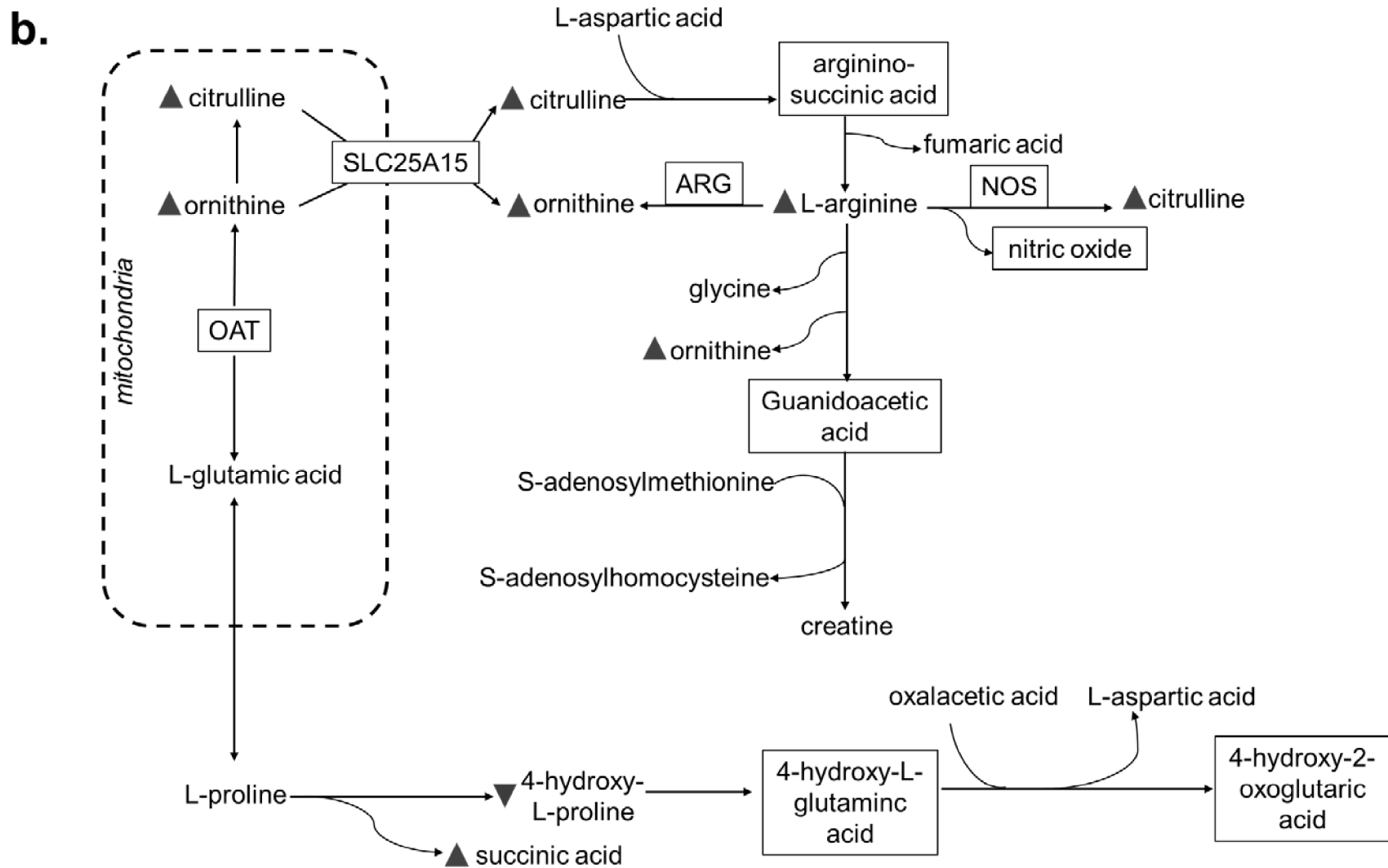


Figure S7. Bile acid synthesis and intracellular arginine and proline metabolism, simplified version (based on the Small Molecule Pathway Database). (a) Bile acid synthesis. Chenodeoxycholic acid is a primary bile acid as it is directly synthesized from cholesterol in liver. After being synthesized, chenodeoxycholic acid is then secreted into bile and intestinal tract, where it is converted by intestinal bacteria to lithocholic acid, which is a secondary bile acid. To increase its solubility and decrease toxicity, lithocholic acid is conjugated with glycine to form glycolithocholic acid. Cholic acid is another primary bile acid. In the intestinal tract, cholic acid is

converted to deoxycholic acid, a secondary bile acid. We found that higher levels of chenodeoxycholic, deoxycholic, and glycolithocholic acids were associated with the presence and severity of CAC. (b) Intracellular arginine and proline metabolism. Arginine/proline metabolism is one of the central pathways for the biosynthesis of arginine and proline from glutamine. We found that arginine/proline metabolism was independently associated with CAC status. Compared to controls, cases had higher levels of L-arginine, ornithine, citrulline, and succinic acid and lower levels of 4-hydroxy-L-proline ($p < 0.05$). Assuming intracellular metabolism of these amino acids mirrors their extracellular metabolism and serum levels, their directionality seems to suggest that there was an increased synthesis of L-arginine and decreased metabolism of proline in participants with CAC.

Note: Metabolites that either were not measured or had a $CV \geq 30\%$ are in boxes. The rest are metabolites analyzed. Total cholesterol level was measured separately in fasting blood samples on a non-HD day. Arrowheads ($\blacktriangle \blacktriangledown$) indicate the directionality when comparing the cases with controls ($p < 0.05$). Abbreviations: OAT, ornithine aminotransferase; SLC25A15, mitochondrial ornithine transporter 1; ARG, arginase; NOS, nitric oxide synthase.

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